Fitness for the Ark: Are zoo bred amphibians ready to go back to the wild?

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Abstract

Zoos are playing an important role in the management of endangered species through the reintroduction of captive-bred animals into the wild. Reintroduction is becoming a high-profile management tool for many threatened species, but it is unknown how many generations of captive breeding can influence a species' physical and behavioural characteristics. Considerable difficulty has been encountered in the reintroduction of endangered species, and genetic adaptations to captivity reducing fitness in the wild are one of several possible reasons for this low success rate. Evaluating the level of fitness and behavioural traits of captive animals could improve the success rates of such programmes.

This PhD project implements a multidisciplinary approach to explore and understand the effects that captivity has on different aspects of the golden mantella frog's ecology and behaviour. During this study we used behavioural observations, microbiology, bioacoustics, playback experiments, and spectroscopy analysis of captive and wild frogs to understand the consequences of being born and reared in a captive environment. Specifically, we examined aspects of our species biology that affect an individual's survival skills and discuss the consequences of this in the long-term for captive populations and for their future reintroduction.

It was observed changes on all the study aspects, skin colouration, body condition, vocalizations, anti-predator responses, skin associated bacteria and species recognition. The results obtained during this study show that captivity does have a significant impact on the behaviour and ecology of this species. However, the results cannot be extrapolated as a general captive effect. Individual frogs at the Mitsinjo breeding centre, when compared to parameters obtained from wild individuals, presented more significant effects on body condition, skin colouration, anti-predator response than Chester Zoo's animals. On the other hand, vocalization from mantellas kept at Mitisinjo had a greater degree of similarity with

wild frogs than Chester Zoo's animals. One interesting result was the fact that wild individuals could still recognize the calls recorded from Chester Zoo animals, regardless of the changes observed.

Animal husbandry seems to play an important role in attenuating or increasing these negative consequences, since not all captive populations showed the same effects. From this it was possible to conclude that captive breeding can only be viable option for the conservation of threatened amphibians, if the necessary care is taken regarding husbandry protocols. Extreme care should be taken regarding husbandry to fulfil the environmental and behavioural needs of each species. The next stage for this research would be to test if a soft-release reintroduction protocol would work as a mitigating measure. If, during a soft release, the behavioural and morphological differences observed between captive and wild individuals diminished, then it could be a time and cost effective measure to solve negative captivity induced changes in amphibians.

Chapter 1 – General Introduction

The concept of keeping animals in a zoo is not new. Rulers in ancient Egypt, China and Rome and European royal families of the Middle Ages had collections of exotic animals for their entertainment (Roe et al., 2014). These early "zoos" existed to please visitors and little consideration was given to the needs of the animals, which were taken from the wild. Modern zoos have progressed a long way from this view (Roe et al., 2014).

From the 19th century to the end of the 20th century, zoos have evolved from menagerie type collections into conservation centres (Roe et al., 2014). The word "menagerie" is used to describe old fashioned zoos that were designed mainly to display a large number of animal species (Kohl, 2004). The aim of the zoos in those days was to display as many interesting species as possible. Most animals were not bred in captivity but taken directly from the wild. The animals were displayed in small cages, without considering the welfare of the animals (EAZA, 2013).

In the 21st century zoos and aquaria want to educate their visitors about the natural world (Roe et al., 2014; WAZA, 2005). Their aim is to ensure that all visitors are aware of the importance of wildlife conservation (EAZA, 2013). The animals in zoos and aquaria should be ambassadors for animals in the wild by inspiring visitors to act for conservation, caring for natural ecosystems, understanding the processes that lead animals to extinction by acting more sustainably in their everyday lives. Good modern zoos have four main functions: Conservation, Education, Research and Recreation (WAZA, 2005).

- RECREATION: To provide enjoyment and enrichment for visitors through close contact with living animals.

- EDUCATION: To increase the level of awareness, knowledge and understanding of visitors about animals, the environment and conservation, and to motivate behaviour change, which will help the environment.
- RESEARCH: To conduct and facilitate research on animals both in captivity and in the wild, with particular emphasis on threatened species.
- CONSERVATION: To be involved in programs that assist the survival of wild populations of animals. This is often done in partnership with other organisations.

Zoos and aquariums can operate across the whole spectrum of conservation activities, from: *ex situ* breeding of threatened species; research, public education, training and influencing and advocacy; through to *in situ* support of species, populations and their habitats; through their exceptionally large "captive audience" of visitors whose knowledge, understanding, attitude, behaviour and involvement can all be positively influenced and harnessed (WAZA, 2005).

One of the main functions of modern zoos is an active involvement in the conservation of endangered species (Roe et al., 2014). Zoos can have many roles when it comes to contribution to conservation including: development of new techniques, fundraising for *ex-situ* projects, capacity building of local NGOs in developing countries, supplying of animal management experts, managing their populations in *ex situ* breeding programmes to create safety net populations and, by being involved in reintroduction programmes (EAZA, 2013; Roe et al., 2014). In addition to this, all research conducted in zoos is vital for conservation and the understanding of biodiversity (EAZA, 2013).

Animals kept in breeding centres can function as significant demographic and genetic reservoirs from which wild populations can get vital infusions to secure a declining

population or to found a new population (Rahbek, 1993). Captive breeding may be the short term practical conservation option for species confined to dwindling habitats (Frankham, 2008; Conway, 2011). A striking example is the increase of amphibian collections in zoos as a response to chytridiomycosis crisis: a fungal infection responsible for precipitous global amphibian population declines (Conde et al., 2011).

Zoological parks are playing an increasingly important role in the conservation of endangered species through the reintroduction of captive-bred animals into the wild (Bloxam & Tonge, 1995). However, *ex situ* programmes are often criticized for their low success rates and high costs (Bloxam & Tonge, 1995). Different studies have showed that captive populations of wildlife are likely to adapt quickly to their captive environment, which may significantly change the phenotype of the population and reduce the ability of captive-bred individuals to survive and reproduce in the wild (Gilligan & Frankham, 2003; Connolly & Cree, 2008; Frankham, 2008; Griffiths & Pavajeau, 2008; Sinn et al., 2013; Harding et al., 2015). Besides keeping a breeding population of endangered species, is necessary to also evaluate the animals' behavioural skills necessary for releasing and subsequent survival of these animals kept in captivity.

Captivity can lead animals to express abnormal behaviour and reduce wild conspecifics recognition (Sun & Narins, 2005), which can, often limit the success of subsequent reintroduction attempts (Balmford et al., 1996; Kraaijeveld-Smit, 2006). It is therefore necessary to evaluate the level of fitness of captive-born individuals. Some previous studies on the effects of captive breeding on fitness have generally focused on model organisms or species of commercial importance (e.g. *Drosophila*, Reed et al., 2003; Gilligan & Frankham 2003; salmon, Säisä, Koljonen & Tähtinen, 2003; Fox & Heath, 2003). Such studies have not explicitly addressed behavioural traits that influence ecological interactions

between species in the wild such as identifying prey and avoiding predators (Kraaijeveld-Smit, 2006).

Management, conservation and recovery plans for endangered or threatened amphibians frequently involve repatriation, relocation, or translocation programs (Dodd & Seigel 1991, Germano & Bishop 2008). A number of traits make amphibians good candidates for captive-release programs, including high fecundity, lack of parental care and many small sized amphibian species can be bred in captivity in a very cost-effective manner (Bloxam & Tonge 1995, Harding, Griffiths & Pavajeau, 2015). Reintroduction of amphibians has been criticized on the grounds that these programs have poor planning and monitoring and for the lack of results showing the creation of a self-sustaining population in the long-term (Bloxam and Tonge 1995). In a review of amphibian and reptile translocations, Dodd and Seigel (1991) found that amphibian projects have very low success rates, especially when compared to translocations of other taxa, however, a more recent review conducted in 2008 showed that the success rate of amphibian and reptile translocations reported over the period between 1991 and 2006, was twice that reported in an earlier review (Germano & Bishop 2008). A more recent study by Harding, Griffiths and Pavajeu (2015) shows that since the release of the "Amphibian Conservation Action Plan" in 2004 there was a 57% increase in conservation programmes focused on amphibians and a higher success rate, with previously failed reintroductions now being successful. Despite the considerable effort that has been devoted to strategic planning for amphibian conservation, and the increase in success rates of amphibian reintroductions, the current figures demonstrate that there is still room for significant improvement, and the necessity to add pre-release screening.

Reintroductions are costly and time consuming, therefore, to make the best use of resources available it is important to screen individuals prior to release not only for health checks but also important behavioural traits (Canessa et al., 2016). Checking the behavioural responses of the involved animals could potentially increase the success rate of release programmes (Kraaijeveld-Smit et al., 2006; Teixeira et al., 2007). Only a few studies involving reintroduction of herpetofauna show data on any kind of fitness or behavioural skills evaluation for captive-born animals (e.g., rattlesnakes, Chiszar et al., 1993; Indian iguanas *Cyclura* sp., Alberts et al., 2004). If captive animals are to be released into the wild, these issues must be taken into account (Germano & Bishop, 2008).

To demonstrate the size of the knowledge gap on pre-release screening of captive amphibians for reintroduction, a search using "Web of Science" for relevant literature using the following terms: reintroduction or captive breeding in combinations with the terms amphibian, frog, salamander, newt, toad and caecilian was used to assess this information. After excluding repetitions, papers that did not involve a captive element, articles that did not have an abstract and whose titles were not obviously related to the topic, a total of 285 papers were assessed on what type of screening captive individuals were going through. We also excluded programs where captive breeding or translocation was carried out for commercial or medical purposes or to resolve human–wildlife conflict that did not have conservation as an explicit goal (Germano et al., 2015). Screening types were divided into the following categories: behaviour, genetics, health, reproductive ecology, hormones and microbiome. A total of 76 papers presented some form of screening, among these some presented multiple sorts of screening. Only four papers presented some form of behaviour evaluation while 37 only had a basic health check as part of their screening process. This shows how much amphibians are neglected when it comes to assess the effects of captive breeding on their behaviour, health, physiology and ecology.

The behavioural integrity of wildlife is one the most important aspects to conserve in captive population, especially if animals are part of a conservation programme that will include reintroduction (Schulte-Hostedde & Mastromonaco, 2015). It is important to investigate whether captive breeding centres are providing the appropriate environmental resources and stimuli, which allow species to satisfy their biological or behavioural needs; thereby, also improving their welfare (Young, 2003).

Evaluating the behaviours seen in captivity is one of the greatest challenges faced when keeping animals in captivity (Young, 2003). Different approaches have been suggested to increase natural behaviour expression such as offering animals operant tasks for food to making enclosure structures more ‘‘naturalistic’’ in order to promote naturalistic behaviours (Hosey, 2005). Maintaining natural behaviours by captive animals is an important goal, especially if captive-bred animals are part of conservation efforts (Connolly & Cree, 2008; Germano & Bishop, 2008). Thus, evaluations based on the welfare of the animals should be used in conjunction with wild-captive comparisons if evaluation of well-being is the objective. (Hosey, 2005).

Most animals maintained in captivity are constantly exposed to an environment that vary greatly from the natural conditions found in the wild, which can easily promote phenotypic changes (Slade et al., 2014). An increasing number of studies show that captive breeding can result in rapid selection or plastic responses in phenotypic or life-history traits that can reduce an individual’s fitness on release and compromise the chances of successful reintroduction (Slade et al., 2014). Among the different traits that are important for survival back in the wild is species recognition through visual and acoustics signals, anti-predator defences and defence against pathogens.

Modern zoos and aquaria are trying to instruct their guests about the living world, ensuring that they comprehend the value of wildlife and the importance of nature conservation. But if animals kept at the zoo do not fully represent their wild counterparts, this educational role of zoos could be compromised. Zoos educate their visitors by displaying animals in good exhibits that provide for their physical and psychological needs (EAZA, 2013).

Therefore aim of this research is to answer the following questions using wild and captive populations of golden mantella frogs:

1.1 Do the vocalisations of captive frogs differ from wild ones?

Species recognition is an essential trait for the survivorship of released animals (Kraaijeveld-Smit et al., 2006). Captive animals, if released, should be able to recognize and appropriately respond to their wild conspecifics. Communication is the foundation upon which all social relationships between animals are built (Brumm & Slabberkoorn, 2005). Vocalizations are shaped by the acoustic complexity of the environment individuals are living, and man-made acoustic sounds affect anuran chorus behaviour by modulating call rates of the chorus participants (Sun & Narins, 2005). It is easy to perceive that the acoustic environment from a zoo will differ greatly from the background noise heard in a forest.

Acoustic communication plays a fundamental role in reproductive behaviour across a wide diversity of mating systems among different species (Bee, 2007). Phenotypic differences, such as call differences could lead to captive animals being more likely to assortative mate with other captive-born animals, which could lead to producing two separated populations of the same species (Slade et al., 2014). Avoidance of captive–wild mattings would compromise the effectiveness of a reintroduction attempt (Slade et al., 2014). The inability of released individuals to communicating with wild individuals could negatively impact any conservation effort involved in a reintroduction programme (Gilligan & Frankham 2003; Mathews et al., 2005).

During this research, we aimed to evaluate if the captive environment has affected the golden mantella frogs' calls and, through the use of playback experiments, investigate whether captivity has also affected the frog's ability to recognise wild conspecifics.

How does the body condition of captive frogs compare with wild ones?

Body condition is a simple index that could be used to infer about the health state, stress levels and many more aspects of the individuals behaviour (Maccracken & Stebbings, 2012). Body condition is a measure of the energetic (or nutritional state) of an individual animal, especially the relative size of energy reserves such as fat and protein (Peig & Green, 2009). A variety of disease conditions attributed to nutritional deficiency or excess have been reported in amphibians in breeding programs (Ferrie et al., 2014). Low body condition could also be a consequence of long exposure to stress sources (Morgan & Tromborg, 2007).

Wild amphibians feed on a variety of invertebrates, with quite variable nutrient composition (Lima et al., 2010). In addition, the food within the invertebrates' gastrointestinal tract and the material clinging to their exoskeleton (such as soil and pollen) also adds to the variety of nutrients consumed in the wild (Ferrie et al., 2014). Meanwhile, ex situ managed insectivorous amphibians are fed a much smaller variety of commercial feeder insects and other invertebrates (Livingston et al., 2014). Trying to mimic such a diverse diet in captive is one of the major challenges when keeping frogs in captive (Livingston et al., 2014).

Captivity can present many sources of stress (Mason, 2010) from which animals cannot escape or manage it (Morgan & Tromborg, 2007). The internment state of stress could lead to a chronic level of stress (Mason, 2010), having negative consequences on the growth rate (Chrousos, 1997; Tsigos & Chrousos, 1995), body weight (Bartolomucci et al., 2004; Konkle et al., 2003), and food consumption (Schumann et al., 2014) all these leading to a lower body condition.

On the other hand, the lack of space and physical challenges in a captive environment combine with the food availability and no competition could easily lead animals to an obesity

state (D'Eath et al., 2009). As for humans, obesity can increase the chances of diseases and compromise mobility (Miranda-Anaya et al., 2016).

Underweight animals probably would not have enough energy reserves to cope with the challenges of finding shelter, food or escaping predators in a new environment after a reintroduction (Teixeira et al., 2007). While obese individuals, might not be agile enough to escape predators or compete for territory (Miranda-Anaya et al., 2016).

During this research we aimed to compare the body condition of different captive and wild populations of golden mantella frogs to observe the effects of a captive diet and environment on their body condition and, how this could be related to other behavioural traits.

1.2 Does antipredator behaviour of captive frogs differ from wild ones?

Captive environments are highly predictable and the lack threatening situations could lead to important defensive responses weakening or disappearing during generations of captive breeding (Kraaijeveld-Smit et al., 2006, Teixeira et al., 2007). As a response to this loss of predators, antipredator behaviours are often lost (Blumstein et al. 2006), making reintroduced animals more vulnerable to predation after being released reducing the effectiveness of conservation programmes (Mesquita & Young, 2007).

For the conservation of a species it is necessary to have information about its behaviour; this is especially important when captive bred animals are to be released into the wild (Shumway, 1999). One of the most important abilities for an animal destined for release into the wild is to know how to respond appropriately to its predators (Kraaijeveld-Smit et al., 2006; Mesquita & Young 2007, Brown & Laland, 2001). Research shows that animals bred and reared in captivity for many generations may lose their ability to recognise predators, due to the relaxing of natural selection (Blumstein et al., 2006). Predator recognition will be most successful if an animal has already been exposed to some threatening situation or anti-predator behavioural training that could retrieve or increase the pre-existing elements (Mesquita & Young, 2007). Antipredator training, therefore, may be a valuable addition to reintroduction programs (Maloney & McLean, 1995) and could be a useful strategy to increase translocation success (Azevedo & Young, 2006; Mesquita & Young, 2007; Teixeira et al., 2007). The remaining question is: Do captive bred golden mantella frogs still have their natural anti-predator response even after several generations in captivity? We have aimed to answer this question using a tonic immobility test.

1.3 How does the colour of captive frogs compare with wild ones?

Brightly coloured signals and ornaments are, with the exception of aposematism, thought to evolve through sexual selection and to provide advantages for attracting mates and intimidating rivals (Macedonia et al., 2014). More colourful males are expected to have increased fitness, either because colour traits are favoured by mate choice or colour functions as a signal of status in intra-sexual competition. Communication via colour traits will be evolutionarily stable when the colour reflects performance, condition or genetic quality of the animal, and colour can thus be considered an honest signal (Plasman et al., 2015).

When compared to wild individuals, many zoo frogs display a faded colouration, (Brenes-Soto & Dierenfeld, 2014). This change in skin colour could be related to diet, carotenoid based colorations in many amphibian species are a good example (Brenes-Soto & Dierenfeld, 2014; Ogilvy, Preziosi & Fidgett, 2012). One of the difficulties faced while keeping frogs in captivity is replicating their natural diet and balancing nutrient intake (Livingston et al., 2014), which can directly affect amphibian skin pigmentation (Brenes-Soto & Dierenfeld, 2014). Environmental aspects could also be related to the change on the skin's colouration. It has been showed that UV light can have an impact on amphibians colour (Michaels et al., 2014). Changes in colouration may affect species recognition and consequently, have negative effects on health and reproductive output by not individuals not being recognize as breeding partners or having their fitness perception altered (Brenes-Soto & Dierenfeld, 2014).

Skin colouration can be affected by many different factors, during this chapter we aimed to quantify the differences in skin colouration of captive and wild golden mantella frogs and understand which factors (e.g. body condition) were involved.

1.4 Does the skin microbiota of captive frogs differ from wild ones?

Amphibian conservation goals depend on effective disease-treatment protocols (Woodhams et al., 2012), becoming an important factor when planning a reintroduction. Amphibians' complex immune system has skin as prime physical barrier. It provides a moist and nutritive substrate for resident skin bacteria, which, in turn, contributes to inhibit the growth of infectious diseases including the *Batrachochytrium dendrobatidis* (Culp et al., 2007). A wide variety of studies have consider the microbiome resident on amphibian skin symbiotic (Costa et al., 2016).

Amphibians in the wild gain skin bacteria through environmental transmission and through interactions with conspecifics and other species (Nyholm et al., 2000; Walke et al., 2011). Whereas in captivity, frogs interact with fewer individuals and less species, therefore, they receive lower exposure to a variety of bacteria, and consequently support a simpler cutaneous bacterial community structure in comparison to wild counterparts, making them less resistant to disease on reintroduction to the wild. (Antwis et al., 2014a). Captivity also provides a less diverse environment through which to gain bacteria when compared to the complexity of a natural habitat (Walke et al., 2011). Animals in the wild are used to being exposed to a wide range of microorganisms and parasites that captive animals do not experienced (Walke et al., 2011).

Another way in which wild amphibians can obtain their bacteria in their skin is through vertical (parental) transmission (Walke et al., 2011). This sort of social immunity could help protect the eggs and, consequently provide important bacteria as pre-adaptation against pathogens and parasites from their natural habitat (Cotter & Kilner, 2010). While in captivity, many times, eggs and tadpoles are kept in a different tank, which would prevent this microbiome transmission. Different studies have already shown the negative

consequences of captivity for the amphibians' microbiome richness and diversity (Antwis et al., 2014a; Becker et al., 2014; Sabino-Pinto et al.; 2016). During this research we aimed to evaluate if the skin microbiota found on captive and wild golden mantella frogs are significantly different, and if so, quantify this difference.

1.5 Conclusion

By answering the aforementioned five aims, I hope to provide knowledge on how the captive environment can affect different aspects of the golden mantellas ecology, and help answer the main question posed by this thesis as to whether golden mantella frogs bred in captivity are suitable candidates for reintroduction programmes.

1.8 Study Species

The Golden Mantella Frog (*Mantella aurantiaca)* (Figure 1) is a species classified as critically endangered by the IUCN (Vences & Raxworthy, 2004) and is endemic to the Moramanga district, in the Region of Alaotra-Mangoro, Madagascar (Figure 2). It is well known due to its aposematic orange-red colouration and presence in the international pet trade (Edmonds et al., 2015). Potential predators for the species would be reptile species such as *Zosaurus madagascariensis* and *Tamnosophis lateralis* (Jovanovic et al., 2009).

Figure 1. A wild specimen of Golden Mantella frog (*Mantella aurantiaca*) observed in Madagascar (Photo by Luiza Passos).

The Golden Mantella has a distribution (Figure 2) restricted to a fragment of forest surrounded by degraded land, and the remaining forest is under threat from subsistence agriculture, timber extraction, and over-collecting for commercial purposes. A significant proportion of its population is located inside or near the area of the Ambatovy mine (Vences & Raxworthy, 2004; Randrianavelona et al., 2010). Following a conservation needs

assessment, the Amphibian Ark prioritised *M. aurantiaca* as a species in need of *ex situ* assistance to ensure its survival (Johnson, 2008; Randrianavelona et al., 2010; Vences & Raxworthy, 2004).

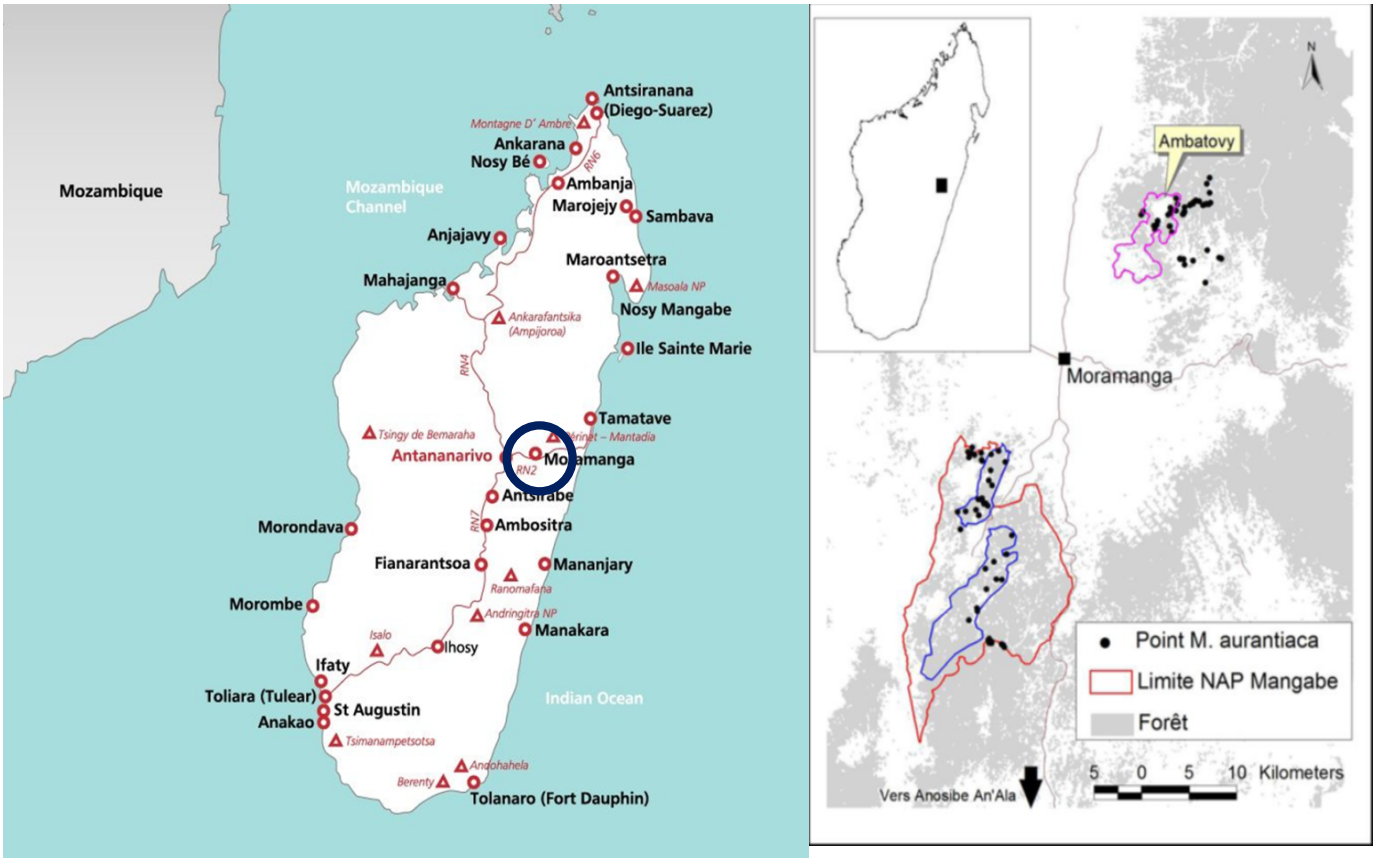

Figure 2. Distribution map of the Golden Mantella frog in the wild (Source: Madagasikara Voakajy)

1.9 Study sites

Mitsinjo Association captive breeding centre:

Association Mitsinjo, a community-run conservation organization, was formed in 1999 by residents of the village Andasibe in east-central Madagascar, an area particularly rich in frog species. While the area supports a tremendous richness of amphibian species, they are also under extreme threat. Habitat loss is the largest cause for concern. Emerging infectious diseases, collection for the pet and food trades, and the ongoing effects of climate change are contributing to declining amphibian populations. In April 2011 it was launched as Madagascar's first biosecure facility (Figure 3) to safeguard amphibians from extinction. Through a contract with the Direction Générale des Forêts and the IUCN/SSC Amphibian Specialist Group of Madagascar, it currently keeps eight local species including Golden Mantella frog taken from the wild (i.e., genetic founders), offspring from which are intended for introductions at an artificially created breeding pond. Animals are kept in tanks with aquarium gravel as substrate, a plant pot, water, coconut shells for hiding. No UV light was supplied. Animals were fed a variety of live invertebrates, but no food supplementation is given. During this project only data from founder and their offspring (F1) were collected. In addition to maintaining the Golden Mantella, the breeding centre also studies the captive care requirements of other poorly known species.

Figure 3. a) General view of the Mitsinjo Biosecure facilities and b) close-up view of one the golden mantella frog tanks (Photo by Luiza Passos)

Mangabe Rainforest:

The rainforests of eastern Madagascar are legendary for their high endemism rates and biodiversity. Mangabe forest, or the 'blue forest (Figure 4), a site of international biodiversity, covers approximately 40,000 ha in eastern Madagascar and is divided between two administrative districts, Moramanga in the north and Anosibe An'ala to the south. The protected area provides essential habitat for the critically endangered golden mantella, whose entire range covers less than 35 square miles. Over 60% of the remaining golden mantella population are found on Mangabe according to recent studies on high conservation priority sites for mantella frogs. Other endangered wildlife found in the proposed reserve includes the Aye-aye (*Daubentonia madagascariensis*), a nocturnal lemur; the Fossa (*Cryptoprocta ferox*), a cat-like carnivore; the Tarzan Chameleon (*Calumma tarzan*); a gecko, *Phelsuma pronki*; and two species of bats, the Madagascan Fruit Bat (*Eidolon dupreanum*) and the Madagascan Flying Fox (*Pteropus rufus*). Due to low agricultural yields, slash-and-burn agriculture is commonly employed to clear and prepare new farmlands within Madagascar's Moramanga District. As the human population increases its forests are being quickly transformed into fields. Likewise, endangered wildlife faces greater danger from more hunters. In Madagascar's Moramanga District these threats pose a dire risk to endemic wildlife, including the golden mantella. Data sampling for this study was done in the Moramanga region.

Figure 4. General view of Moramanga forest a sample site for Golden Mantella frogs, Madagascar (Photo by Luiza Passos)

Ambatovy Mining Site:

Ambatovy's Mine is located within a species- rich region of Madagascar at the southern end of the remaining Eastern Forest Corridor at Moramanga region (Figure 5 and 6). As a responsible mining enterprise, Ambatovy adheres to stringent environmental standards. Ambatovy's Environmental Management Plan (EMP) provides the framework that ensures that all issues identified during the Environmental and Social Impact Assessment (ESIA) are addressed through appropriate mitigation and monitoring. As part of the Environmental Management Plan, there is a Conservation zone of native forest kept in pristine conditions by the mining company and species inventories and translocation of live animals to conservation forest refuge areas called the Receptor ponds prior to clearance.

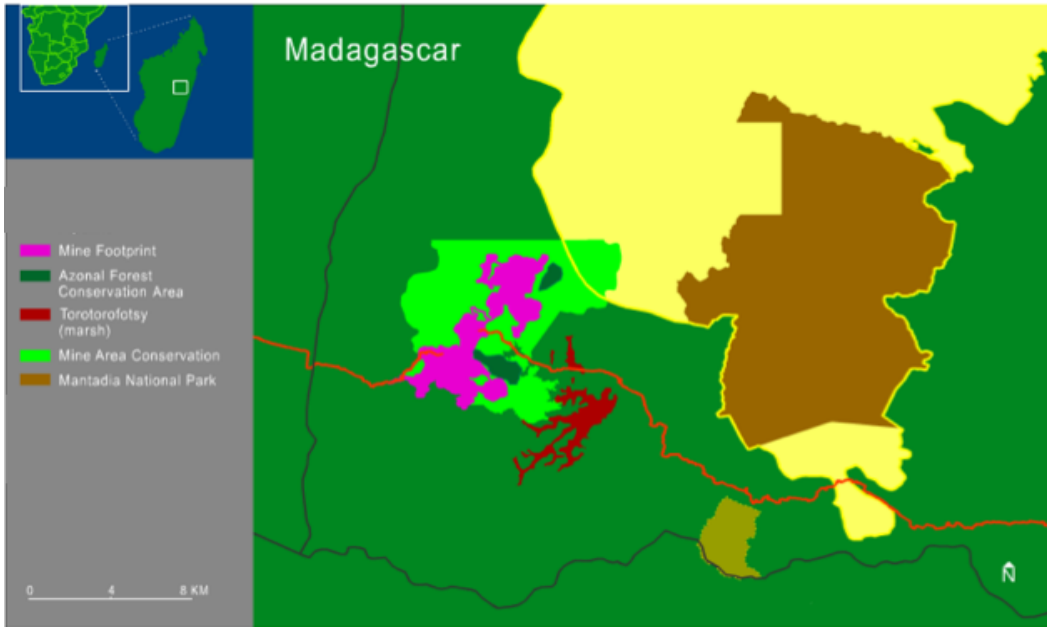

Figure 5. Map showing Ambatovy mining area, conservation area and national parks (Source: http://www.ambatovy.com)

Figure 6. Golden mantella habitat at Ambatovy mining conservation zone (Source: http://www.ambatovy.com)

Chester Zoo:

Chester zoo is one the largest zoos in Europe and is actively involved in the conservation of the Golden Mantella frog in Madagascar. The zoo currently maintains two *ex situ* populations of *M. aurantiaca*, one is on public display at the Tropical Realm exhibit (Figure 7) and a second group is kept off show in a biosecurity container specifically for breeding and research (Figure 8). No exchange of individuals took place between groups for the duration of the study. The biosecurity container is kept under temperature and humidity regimes to give the frogs a similar environment as they would experience in the wild. Frogs are kept in naturalistic tanks with different live species of plants, moss for substrate, water, hiding places under rocks, UV light and heaters to mimic the natural conditions found in Madagascar. Enclosures are annually modified to keep animals under rainy and dry periods as per their natural environments.

Figure 7. External view of the Golden Mantella frogs' enclosure at the Tropical Realm exhibit, Chester Zoo, UK (Photo by Nathan Wright)

Figure 8. The biosecurity container at Chester Zoo in which golden mantellas are kept. (Photo by Gerardo Garcia)

1.10 Ethical Approval

All the research reported in this study was approved by the Chester Zoo's Ethics Committee, University of Salford Science and Technology Ethics Panel (ST1617-82) and, it conforms to all regulations and laws in all relevant countries in relation to care of experimental animal subjects. Furthermore, we can confirm, from our post-experimental monitoring, that no animals suffered any injuries, became ill or were negatively affected as a result of this study. Furthermore we followed the Association for the Study of Animal Behaviour's Guidelines for the care of animals (ASAB, 2014).

Chapter 2 –Neglecting the call of the wild: Captive frogs like the sound of their own voice

* This chapter has already been published at PlosOne (Passos, L.F., Garcia, G. & Young, R.J., 2017a. Neglecting the call of the wild: Captive frogs like the sound of their own voice. *PloS one*, 12(7)) See appendix.

2.1 Abstract

Acoustic communication is highly influential in the expression of social behaviour by anuran amphibians, transmitting information about the individual's physical condition and motivation. We studied the phonotactic (approach movements) responses of wild and captive male golden mantella frogs to conspecific wild and captive playback calls to determine the impact of captivity on social behaviour mediated by vocalisations. Calls were recorded from one wild and two captive populations. Phonotaxis experiments were then conducted by attracting *M. aurantiaca* males across a PVC grid on the forest floor or enclosure floor to a speaker. For each playback, the following parameters were recorded to define the accuracy of phonotaxis: (1) number of jumps; (2) jump angles; (3) jump distances; (4) path straightness. During this experiment we observed that wild frogs had a similar behavioural (phonotaxis) response to calls independent of their source while frogs from Chester Zoo had a significantly stronger response to calls of other conspecifics held separately at Chester Zoo. The lack of appropriate phonotaxis response by captive bred frogs to the calls of wild conspecifics could have serious negative conservation implications, if the captive bred individuals were released back to the wild.

Key words: Phonotaxis, behavioural skills, bioacoustics, playback experiments

2.2 Introduction

Communication is the foundation upon which all social relationships between animals are built (Brumm & Slabberkoorn, 2005). Acoustic communication is probably the most influential trait in the social behaviour of anuran amphibians. Although the circumstances in which animals vocalize vary between species, virtually all male frogs incorporate some form of advertisement call into their vocal repertoire that is usually a necessary precursor to successful courtship and mating (McClelland, Wilczynski & Ryan, 1996).

In anurans significant information about the individual's fitness is transmitted by acoustic signals (Duellman & Trueb, 1996; Ryan, 1988). Among male frogs, vocalisations allow the identification of the resource holding potential of an opponent (Bee et al., 1999), facilitate inter-male spacing (Brenowitz, 1989; Marshall et al., 2003) and permit the recognition of territorial neighbours (Bee, 2007). Field experiments using playback calls have revealed that vocalisations also play an important role in sexual selection during male–male competition and female choice in many species (Brenowitz, 1989; Marshall et al., 2003).

Phonotaxis is defined as any kind of movement or orientation towards specific acoustic signals (Gerhardt & Rheinlaender, 1980). Positive response is taken as evidence of both perception and recognition of the acoustic stimulus by the receiver (Bee, 2007). It has been widely demonstrated that playback experiments are an adequate methodology to analyse phonotactic responses of frogs (Mayer et al., 2014; Narins, 2003; Gerhardt & Rheinlaender, 1980).

It is believed that anthropogenic sounds can significantly affect the vocalisations of animals (Caldart et al., 2016). This could have serious implications for reintroduction programmes (Brumm & Slabberkoorn, 2005). Therefore, we studied the phonotactic

responses of wild and captive male golden mantellas (*Mantella aurantiaca*) to conspecific wild and captive playback calls.

2.3 Methodology

2.3.1 Study subject

The study subject was the golden mantella frog, for more details please see section 1.8.

2.3.2 Study sites

Golden mantellas calls were recorded from three different populations: wild calls from Mangabe (Madagascar) and captive calls from Mitsinjo Captive Breeding Centre (located in Madagascar, only calls from the F1 frogs were recorded and used; no playback experiments were done here and Chester Zoo (UK), more than 7 generation in captivity. The zoo currently maintains two visually and acoustically isolated *ex situ* groups of *M. aurantiaca*, one is on public display at the zoo's Tropical Realm exhibit from which calls were recorded and a second group is kept off show in a biosecurity container specifically designed for conservation-related research, where the playback experiment was conducted with these frogs.

The phonotaxis experiments were performed with wild frogs in Madagascar and from captive frogs kept at Chester Zoo.

2.3.3 Recording Calls

Frog calls were recorded using a digital audio recorder (H4n Handheld Digital Recorder, Zoom USA) with an omnidirectional microphone. Before recording calls, a pilot study was undertaken at the University of Manchester with their captive colony of golden mantella frogs to ensure the microphone and recorder had the appropriate sensitivity (i.e. could record all the frequencies emitted by the subjects). Recordings were analysed for call characteristics using Raven software (Bioacoustics Research Program, 2014) The characteristics analysed were (Figure 9):

1- Call duration (s): Duration from the beginning of a call to its end.

2- Call period (s): Duration from the beginning of a call to the beginning of the next call.

3- Pulse rate: The number of individual components of each call.

4- Interpulse interval (s): Time between the pulses of a call.

5- Dominant frequency (Hz): The frequency with maximum intensity.

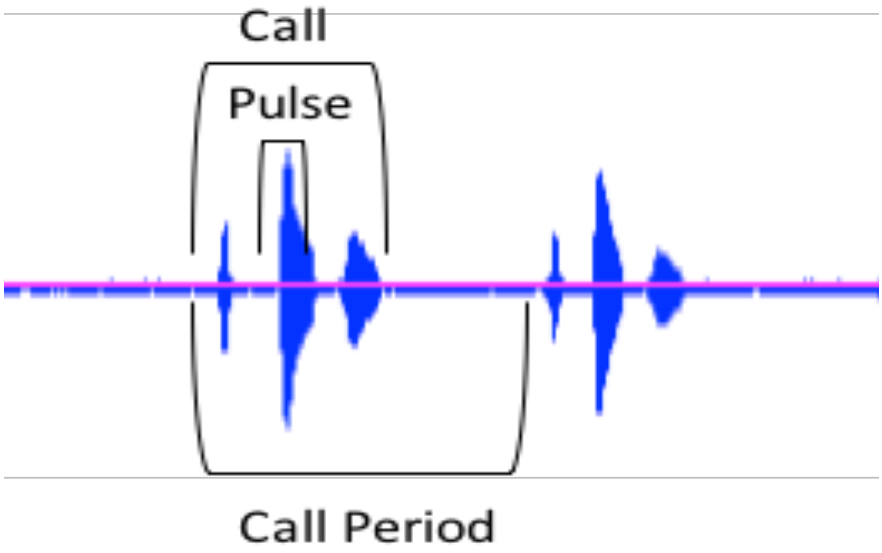

Figure 9. Wild golden mantella frog call waveform showing some measured call characteristics.

We analysed three call sequences of 20 different males *M. aurantiaca* from each population. In addition, to minimize intraspecific variance, we used mean values of the call parameters within and between individuals.

2.3.4 Phonotaxis experiments

Prior to any experimentation, measurements of sound pressure (noise) levels that animals are already exposed to during routine husbandry at Chester Zoo were taken using a sound pressure meter (SIP95 Sound Level Logging Meter FFT Audio Analyser, Balkon Technology) to avoid exposing animals to any extreme acoustic stimuli (Figure 10). Playback recordings were used with similar amplitude (i.e. volume) to what the animals were already exposed to in captive or natural environments. Calls were previously recorded from the three different populations using a digital audio recorder (H4n Handheld Digital Recorder, Zoom USA) with an omnidirectional microphone. Calls were edited for length and background noise using Audacity® (Audacity, 2014) recording and editing software. During the experiment, we recorded the phonotaxis accuracy of a wild (Mangabe) and a captive population (Chester Zoo) of golden mantella frogs to three different recordings (used as treatments): one from a wild population of golden mantellas from Mangabe, and two from captive populations: one from Chester Zoo and one from Mitsinjo. Calls were presented using a randomized block design.

Figure 10. Sound pressure meter inside a golden mantella tank at Chester zoo to measure the sound pressure (noise) levels that animals are already exposed to during routine husbandry (Photo by: Luiza Passos).

In the wild, active males were collected by hand from the ponds and put in a plastic box until the experiment. Frogs were kept in the box for nearly one hour, until they had

recovered from being hand caught and were behaving normally with no signs of acute stress (i.e. abnormal behaviour, tachycardia). Each animal was tested only once. Phonotaxis playback experiments were than conducted by attracting *M. aurantiaca* males across a 100 x 60 cm PVC mat on the forest floor or enclosure floor to a Bluetooth speaker (model HX-P240PK, Jam Plus) broadcasting calls, similar to the method described by Mayer and colleagues (2014). During the experiment, 21 males from Chester Zoo and 39 males from Mangabe had their phonotaxis response tested. Frogs were placed 10 cm away from the mat (see Figure 11). Trials were not scored if males did not enter the board from the front edge of the board. The experiment was videotaped with a Canon PowerShot SX520 HS digital camera.

Previous playback studies with *Allobates femoralis* (Narins, 2003) and *Ranitomeya imitator* (Mayer et al., 2014) revealed that at distances closer than 20 cm to the sound source the animals searched for a visual signal in addition to the acoustic stimulus; taking this in consideration, playback sessions ended when the frog reached within a perimeter of 20 cm of the speaker (Figure 11).

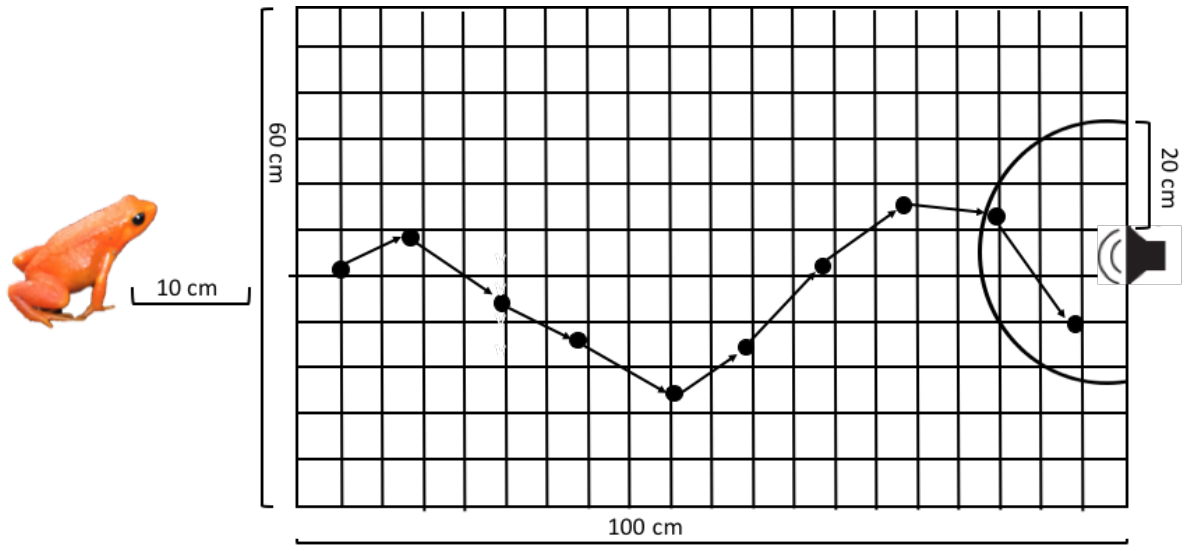

Figure 11. Schematic diagram of a male golden mantella frog when approaching a playback call on a speaker, the grid area is a PVC mat.

2.3.5 Movement Analyses

Each jump of an approaching male was plotted by manually digitizing the recorded videos in a stop-motion view with software called BORIS (Friard & Gamba, 2016). The grid on the mat was used to identify frog positions and for calculating distances between positions and jump angles (Figure 12). Jump angles and distances were measured as soon as the animal had entered the board and until it came within 20 cm of the broadcasting speaker (Figure 13). For each playback, the following physical characteristics of frogs were analysed to define the accuracy of phonotaxis: (1) number of jumps; (2) jump angles (jump angle divergence of the new jump position to the target axis; Figure 12); (3) jump distances; (4) path straightness (summing each jump distance for the path taken by the individual in relation to the straight line from the first entered position to the target); (5) duration (how long, in seconds, the frogs took to reach the speaker). The accuracy of the phonotactic approach was quantified using jump angles and the straightness of the path; values are given as percentage of path length in relation to the straight-line distance. All statistical analyses were done using R Studio (R studio team, 2015).

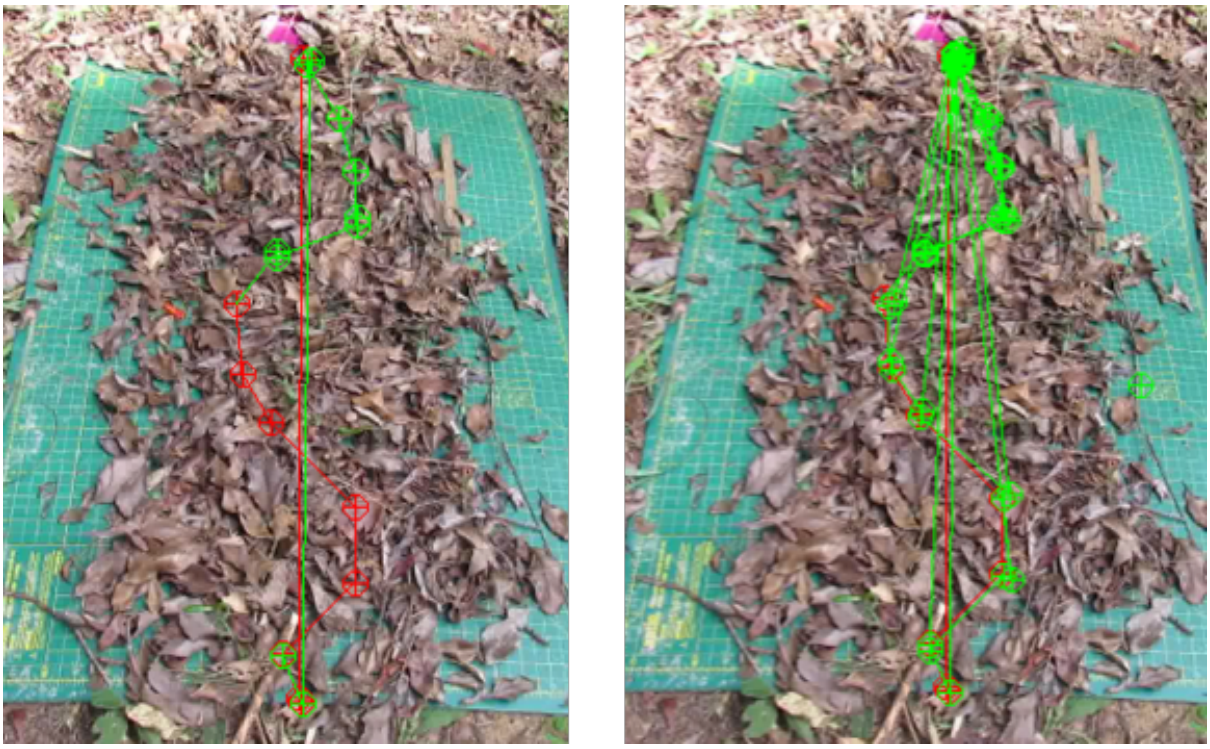

Figure 12. Examples of how the measurements of number of jumps and jump distance was measured (a) and how the jump angles (b) were measured using BORIS software.

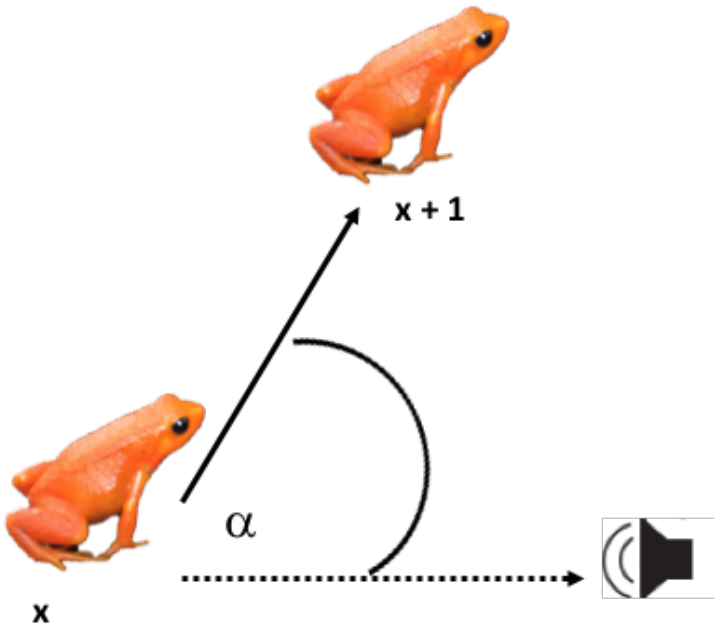

Figure 13. Illustration of how the jump angle of male golden mantella frogs was calculated in a playback experiment. The dashed line indicates the straight line from the frog to the sound source, X the initial position of the frog and X + 1 the measured jump position.

2.4 Results

Call characteristics (Table 1) were compared between the three different populations using one-way ANOVA tests (Table 2). Tests found significant differences between the populations on all the parameters analysed ($p<0.05$). The Tukey *posthoc* test (Table 3) confirmed that calls from Chester Zoo animals were significantly different ($p<0.05$ in all cases) from calls obtained from the wild population on all the analysed characteristics. Vocalisations from Mitsinjo breeding centre were significantly different from Mangabe calls in duration and period ($p<0.05$). Chester Zoo and Mitsinjo recording were statistically different in all parameters except for pulse numbers ($p<0.05$).

Table 1. Call characteristics results for different wild and captive populations of golden mantella frogs.

Population	Location	Duration (s) ± sd	Period (s) ± sd	Pulse rate ± sd	Interpulse (s) ± sd	Dominant frequency(Hz) ± sd
Mangabe	Wild	0.043 ±0.004	0.090 ±0.05	2.92 ±0.27	0.008 ±0.002	4875.00 ±0.00
Chester Zoo	Captive	0.053 ±0.011	0.750 ±0.620	3.90 ±0.72	0.018 ±0.006	5198.01 ±172.84
Mitsinjo	Captive	0.062 ±0.008	0.120 ±0.063	4.04 ±0.19	0.005 ±0.001	4941.96 ±146.25

sd = standard deviation

Table 2. ANOVA results for call parameters comparison between the three samples population, Chester Zoo, Mitisinjo breeding centre and wild animals.

Parameter	F	df	p
Duration (s)	102.51	2, 92	0.001
Period (s)	11.70	2, 44	0.001
Pulse rate	54.17	2, 92	0.001
Interpulse (s)	15.20	2, 185	0.001
Dominant frequency(Hz)	55.54	2, 92	0.001

Table 3. *Posthoc* Tukey test results for golden mantella frogs' call characteristics from different wild and captive populations.

Populations	Duration	Period	Pulse rate	Interpulse	Dominant Frequency
Mangabe x Mitisnjo	$p< 0.01$	ns	$p< 0.01$	ns	ns
Mangabe x Chester	$p< 0.01$	$p< 0.01$	$p< 0.01$	$p<0.05$	$p< 0.01$
Mitisnjo x Chester	$p< 0.01$	$p< 0.01$	ns	$p< 0.01$	$p< 0.01$

Phonotactic experiments resulted in 34 approaches of wild golden mantellas and 21 for the Chester Zoo's frogs (i.e. a total of 55 different individuals). In general, captive frogs took longer and used a lengthier and less accurate path to reach the speaker than wild frogs. All trials with Chester Zoo's frogs resulted in a phonotaxis response, however, five trials (two with Mitsinjo's calls, two with Chester's calls and one for Mangabe's calls) from Mangabe's animals, had no phonotaxis response (i.e. no movement) and were, therefore, not analysed. All successful trials were scored for number of jumps, jump distances, jump angles, path straightness and duration (Figure 14).

Generalised linear mixed models (GLMM) were used to compare the golden mantella frogs' phonotactic movement in response to different playback treatments (see Table 4). Calls were used as fixed factors and location as random factors. Wild individuals' responses to wild calls were used as the species' natural response and this was considered as a reference for an expected reaction towards conspecifics. The wild frogs from Mangabe showed no difference ($p>0.05$) in any of the variables measured for all of the three calls (i.e., wild, or captive) used during the experiment.

Table 4. Results of the Generalized Linear Mixed Models describing the relationship between playback treatment (call sources) and different aspects analysed during phonotaxis experiment with male golden mantella frogs

Population	Call	N	Parameter	Coefficient	p-value
Chester	Mangabe	7	N jumps	-0.04	ns
Chester	Mangabe		Jump angles	17.3	0.004
Chester	Mangabe		Jump distance	0.79	ns
Chester	Mangabe		Path straightness	39.9	0.006
Chester	Mangabe		Duration	10.59	ns
Chester	Mitsinjo	7	N jumps	-0.04	ns
Chester	Mitsinjo		Jump angles	-2.78	ns
Chester	Mitsinjo		Jump distance	2.29	ns
Chester	Mitsinjo		Path straightness	47.1	<0.001
Chester	Mitsinjo		Duration	6.39	ns
Chester	Chester	7	N jumps	0.09	<0.001
Chester	Chester		Jump angles	3.49	<0.001
Chester	Chester		Jump distance	-1.8	ns
Chester	Chester		Path straightness	32.2	0.024
Chester	Chester		Duration	7.08	<0.001
Mangabe	Mangabe	13	N jumps	-0.02	ns
Mangabe	Mangabe		Jump angles	1.27	ns
Mangabe	Mangabe		Jump distance	0.18	ns
Mangabe	Mangabe		Path straightness	-2.43	ns
Mangabe	Mangabe		Duration	6.43	ns
Mangabe	Mitsinjo	13	N jumps	2.15	ns
Mangabe	Mitsinjo		Jump angles	4.98	ns
Mangabe	Mitsinjo		Jump distance	1.53	ns
Mangabe	Mitsinjo		Path straightness	2.47	ns
Mangabe	Mitsinjo		Duration	7.8	ns
Mangabe	Chester	13	N jumps	-0.13	ns
Mangabe	Chester		Jump angles	1.27	ns
Mangabe	Chester		Jump distance	-0.58	ns
Mangabe	Chester		Path straightness	-2.73	ns
Mangabe	Chester		Duration	9.19	ns

Chester Zoo's frogs had significant differences ($p<0.05$) in the number of jumps and duration to the speaker when their own call was presented, jump angles for Mangabe and zoo calls, and path straightness between all calls (Table 4); however, different calls had no impact on jump distance ($p>0.05$). Despite frogs making a significantly higher number of jumps to reach the target, phonotaxis accuracy was higher for calls recorded at Chester Zoo with a straighter, shorter and faster path to the speaker (Figure 14). Path straightness when Mangabe's calls were played, resulted in a longer path in relationship to the path used during Chester Zoo calls, and an even longer path was used for Mitisinjo's playback calls.

When the responses of both populations were compared using a t-test (Table 5) all the parameters were statistically different ($p<0.05$), except for jump distance. Wild frogs had a straighter, shorter and faster route even though they made shorter jumps (Figure 14).

Table 5. Student t-test results of the movement analysis of phonotaxis response between wild and captive golden mantella frogs

Location	Parameter	Mean	SEM	t	N	p-value
Wild	N jumps	8.04	0.18	1.97	55	0.02
Captive	N jumps	8.64	0.23			
Wild	Jump angles (°)	51.79	3.17	2.54	55	0.04
Captive	Jump angles (°)	42.62	1.72			
Wild	Jump distance (cm)	11.74	0.68	0.47	55	0.55
Captive	Jump distance (cm)	11.37	0.38			
Wild	Path straightness (%)	49.44	0.45	12.09	55	0.001
Captive	Path straightness (%)	10.33	2.99			
Wild	Duration (s)	49.18	2.00	3.15	55	0.001
Captive	Duration (s)	60.11	2.83			

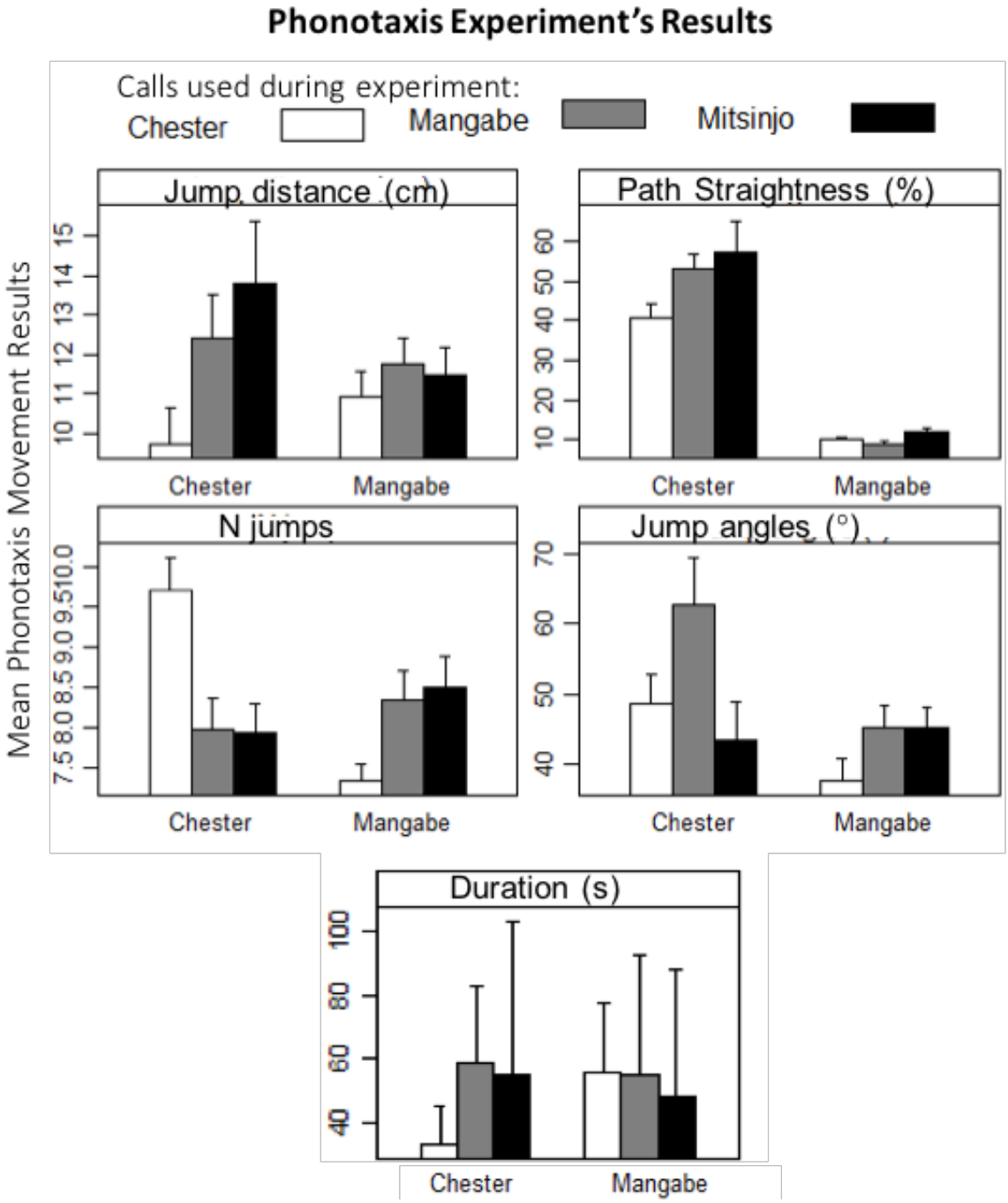

Figure 14. Summary of phonotactic movement results of golden mantella frogs from Chester Zoo and wild frogs (Mangabe) towards playback calls recorded from three different population (Chester Zoo and Mitisinjo captive populations and Mangabe, wild populations. Error bars show ± standard error of the mean.

2.5 Discussion

The analysis of different call parameters showed that calls from Chester Zoo's frogs were statistically different from wild frogs' vocalisations in all analysed characteristics. Whereas the call analyses from the colony held at Mitsinjo breeding centre showed greater similarities to the wild conspecifics. The implication of the observed differences could be negative in terms of reproduction if captive frogs were to be released to the wild. The breeding behaviour of golden mantella frogs involves males calling to court the females with multiple males vocalising simultaneously (Edmonds et al., 2015). Males with calls modified by captivity, if reintroduced, could have their ability to attract females compromised.

Vocalisations are moulded by the acoustic environment in which the species is found (Scofield et al., 2011; Caldart et al., 2016). A zoo's environment has different background noises from sources such as heaters, air filters and visitors, which will lead to a different acoustic complexity (soundscape) than wild habitats. It has already been proved that anthropogenic sounds can alter the calling behaviour of anurans by causing males to modulate their call rate or call frequency (Brumm & Slabbekoorn, 2005; Sun & Narins, 2005). Animals being kept in captivity for many generations could have their calls significantly affected by their environment, while frogs that have been in captivity for only one generation, would not be so affected. This would explain the results found on the call parameters of the Mitsinjo frogs, which had greater similarities with wild calls, while Chester Zoo animals had calls that were significantly different.

During the phonotaxis experiment we observed that wild frogs had a similar behavioural (phonotaxis) response to calls of conspecifics independent of their source (i.e. wild versus captive) while frogs from Chester Zoo had a significantly stronger response to their own calls. Wild frogs had more accurate response, reaching the speaker using a shorter path and in less time while captive frogs were using a longer path and more time, even

though they had longer jumps. It is important to notice that wild frogs would recognize and react in a similar way to captive frogs despite the changes found in their calls. Captive frogs had a weak response to wild calls and, if captive frogs are not able to recognize wild calls or respond appropriately, this could, potentially have negative consequences for breeding success after a reintroduction (Gilligan & Frankham, 2003; Mathews et al., 2005).

The golden mantella frogs breeding behaviour is characterized by groups of males competitively calling to attract females; in this scenario, it is usual to observe males showing aggressive behaviour toward other males as a sign of competition for females. This aggressive behaviour has been described in the wild and observed in captive populations (Slade et al., 2014). The phonotactic response observed in wild frogs corroborate with this premise, while captive frogs only showed this response to their own calls.

Species recognition is a fundamental problem for animals in social contexts (Mathews et al., 2005) for a reintroduction to be successful, released individuals must survive and breed successfully (Gilligan & Frankham, 2003; Slade et al., 2014). Although the accuracy of phonotaxis does not necessarily reflect the accuracy of perception, movement analysis is a powerful approach to examine the auditory abilities of animals (Ursprung, Hödl & Ringler, 2009). When the responses of the two populations were compared, it was possible to observe that frogs from Mangabe (wild) showed a more precise phonotaxis response to calls than golden mantella frogs kept in captivity. Wild male golden mantella frogs would react to defend their territory against all possible opponents presented during the playback experiment, implying that they would recognize conspecific calls even from captive populations.

Animals in captivity are in a confined space in close proximity to other males (Morgan & Tromborg, 2007), which could lead to overlapping territories and to recognition

of individuals as neighbours and not as threats (i.e. “the dear enemy effect”; Temeles, 1994). This would explain the differences observed during the phonotaxis experiment, with captive animals using a longer and less accurate path and, taking longer to reach the speaker. Social recognition is thought to enhance fitness by providing a mechanism that allows animals to direct appropriate behaviours toward specific individuals during repeated social interactions, “the dear enemy effect” (Bee, 2003). Evidence for the dear enemy effect typically consists of a relatively lower level of aggression exhibited by territory holders toward neighbours (Bee, 2003). Dear enemy relationships, however, are not common among territorial species, and several studies have reported that territory residents respond similarly to neighbours and strangers under some conditions (Temeles, 1994).

Frogs characteristically avoid moving unless totally necessary, since it is both energetically costly and increases predation risk (Ryan, 1988). The receiver of an acoustic signal has to judge the sender’s motivational state and adjust its own reaction according to the costs (Ursprung, Hödl & Ringler, 2009). If calls are not perceived as intruders, but as neighbours, it would not trigger such a phonotaxis response. The decision to approach and chase an intruder is, therefore, influenced by the trade-off between fitness costs and benefits (Ursprung, Hödl & Ringler, 2009).

From the results obtained here is not possible to conclude if the captive frogs’ response was due to a dear enemy effect or lack of species recognition, further research is necessary on this matter. Communication can be crucial for breeding success in golden mantella frogs if individuals are being bred for conservation; it is of critical importance to make sure that captive animals, if released, will have the same chances of breeding as their wild counterparts.

Chapter 3 – Do captive golden mantella frogs recognise wild conspecifics calls? Responses to the playback of captive and wild calls

3.1 Abstract

With so many species being threatened with extinction, captive breeding programmes are becoming an important aspect of *ex situ* conservation. Captive populations are important for species conservation and for reintroduction back into the wild. While keeping animals in captivity, some of the most important behaviours to maintain are those associated with sexual reproduction such as courtship and mating. Amphibian reproductive behaviour is associated with call patterns, with studies demonstrating that males advertisement calls elicit positive behavioural responses from females. During this study, we evaluated the response of captive golden mantella frogs to playback calls from different wild and captive populations (one generation in captivity and more than five generations in captivity). During the experiment three different calls were used as treatments: one from wild populations, and two from captive populations. Generalised linear mixed models were used to evaluate the effects of the playback treatments on the behaviour of captive frogs: replicates and enclosures were used as random factors. The model showed that vocalisations from wild individuals led to an increase in movement and social behaviours while calls from captive frogs did not. This was especially true of frogs bred for more than five generations in captivity. This could have negative consequences on the reproduction of captive frogs if released to the wild.

Keywords: bioacoustics, behavioural skills, conservation, amphibians, playback

3.2 Introduction

In the wild, many species are threatened with extinction, thus captive breeding programmes are an important aspect of *ex situ* conservation (Bloxam & Tonge, 1995; Griffiths & Pavajeau, 2008). Maintaining captive populations is not only important in terms of species conservation, but also for potential reintroduction into the wild (Harding, Griffiths & Pavajeau, 2016). One of the main goals of captive animal management is the promotion of natural behaviours and the prevention of abnormal behaviours (Farmer, Plowman & Leaver, 2011) in order to facilitate successful reintroduction programmes (Jule, Leaver & Lea, 2008).

In management terms, some of the most important behaviours to maintain are those associated with sexual reproduction such as courtship and mating (Farmer, Plowman & Leaver, 2011). Amphibian reproductive behaviour is strictly associated with each species' vocalisations (Caldart et al., 2016). For instance, advertisement calls of male frogs are essential to elicit positive behavioural responses from mature females leading to them moving towards preferred signals (i.e. phonotaxis) (Mayer et al., 2014). Acoustic signals convey important information about the sender's fitness (Duellman & Tueb, 1986; McClelland, Wilczynski & Ryan, 1986; Ryan, 1988) and individual reproductive success is directly proportional to calling effort (McClelland, Wilczynski & Ryan, 1996; Witte, Ryan & Wilczynski, 2001; Prohl, 2003).

Playback experiments under field conditions have demonstrated that vocalisations also play an important role in sexual selection during male–male competition in many species (Marshal, Humfeld & Bee, 2003;). For example, among male frogs, vocalisations allow the identification of the resource holding potential of an opponent (Bee, Perrill & Owen, 1999; Edmonds et al., 2015), facilitate inter-male spacing (Marshall, Humfeld & Bee, 2003) and allow recognition of territorial neighbours (Bee, 2007).

The golden mantella frog's breeding behaviour is characterized by males gathering at ponds during rainy season and, competitively calling to attract females. It is usual to observe males showing aggressive behaviour toward other males as a sign of competition for females (Edmonds et al., 2015). This aggressive behaviour has been described in the wild and observed in captive populations (Edmonds et al., 2015).

Animals kept and bred in captivity for conservation purposes such as reintroduction programmes should have a natural behavioural repertoire and be able to recognise wild conspecific calls (Kraaijeveld-Smit et al., 2006). The lack of species recognition is a fundamental problem for animals in social contexts (Kraaijeveld-Smit et al., 2006). During this study, we evaluated the group response of captive golden mantellas frogs to playback calls from different wild and captive populations to verify if a captive colony would recognize wild calls.

3.2 Methodology

3.2.1 Study subject

The study subject was the golden mantella frog, for more details please see section 1.8

3.2.2 Recording Calls

Calls were recorded using a digital audio recorder (H4n Handheld Digital Recorder, Zoom USA) with an omnidirectional microphone. Advertisement calls were recorded, during breeding season, without disturbing animals. Wild frogs were recorded with the microphone positioned 40 cm above the calling individuals. Captive colonies were recorded by putting the microphone on the mesh covers on the top of the tanks, also approximately 40 cm above. Before recording calls, a pilot study was undertaken at the University of Manchester with their captive colony of golden mantella frogs to ensure the microphone and recorder had the appropriate sensitivity (i.e. could record all the frequencies emitted by the subjects). All

populations were recording during the months of January and February 2015, during breeding season.

3.2.3 Study sites

Golden mantella frog calls were recorded from three different populations: wild calls from Mangabe, Madagascar and captive calls from the F1 animals at Mitsinjo captive breeding centre (Madagascar) and Chester Zoo biosecurity container.

3.2.4 Playback experiments

Three different tanks at the biosecurity facilities at Chester Zoo with similar number and sex ratio of frogs were used during the experiment (Tank 1: 11 males, 6 females; Tank 2: 10 males, 5 females; Tank 3: 10 males, 5 females). The playback experiment was done during the month of February 2015, during the breeding season. During the experiment three different recordings were used as treatments: one from wild populations of golden mantellas from Mangabe, and two from captive populations; one from Chester Zoo (more than 5 generations in captive) and one from Mitisnjo (first generation in captive). Calls were edited for length and background noise using Audacity® sound recording and editing software (Audacity Team, 2014). Recording were not edited for any call characteristics such as frequency, number of pulses, calls interval or period. Calls were replicated five times on non-consecutive days to avoid over stimulation and calls were presented using a randomized block design.

Prior to any experimentation, measurements of sound pressure (noise) levels that animals are already exposed to during routine husbandry at Chester Zoo were taken using a sound pressure meter (SIP95 Sound Level Logging Meter FFT Audio Analyser, Balkon Technology) to avoid exposing animals to any extreme situations. Inside the biosecurity containers the main noise sources are water filters, extractor fan, heaters and air conditioners.

Playback recordings were similar in amplitude (i.e. volume, 85.5 dBLin) to what the animals were already exposed to in captivity. The sound-level meter recorded the noise values in decibels every 5 seconds for a 15-min measurement period. A sound level of L_{eq} (Linear weighting) 85.4 dBLin inside the tanks and, 86.4 outside the tanks was observed. Playback experiments were done at similar levels, 85-87 dBLin. Noise levels were measured inside and outside the tank to account for any interference the tanks' wall could have on the sound propagation. During the experiment, Bluetooth speakers (model HX-P240PK, Jam Plus, USA) were placed at a distance of one metre to each tank, calls were played for 10 minutes as a playback stimulus (Figure 15). Speaker frequency response is between 20 and 20000 Hz, the pilot study to choose the microphone showed that the calls were between this ranges.

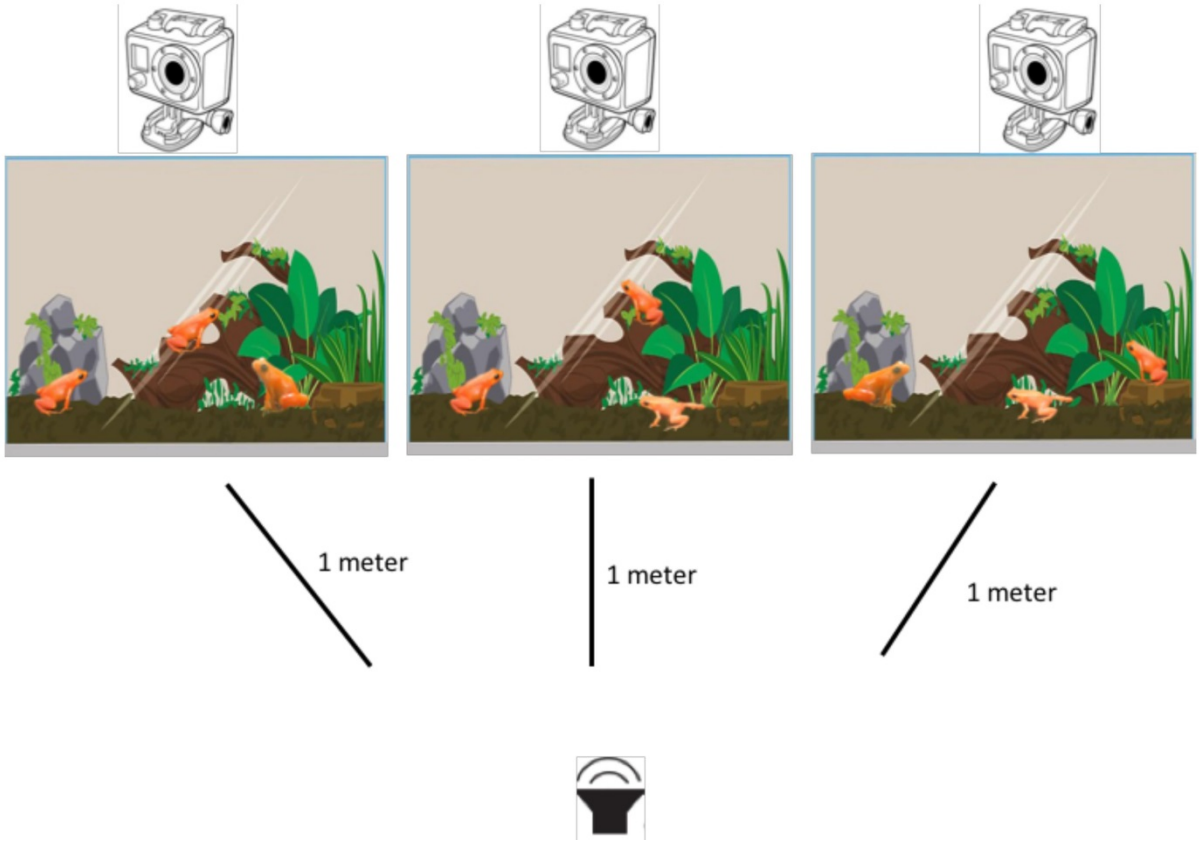

Figure 15. Schematic representation of the playback experiment set up with golden mantella frogs kept in Chester Zoo's (UK) biosecurity container.

The animals' responses were videotaped for 10 minutes before the experiment, during the experiment and for 10 minutes after the playback for behavioural analyses. Playback experiments were always performed during the morning, between 9:00 am and 11:00 am, to match the time golden mantella frogs are active in the wild (Andreone & Luiselli, 2003; Piludu et al., 2003). This experiment was designed to mimic wild conditions: during the breeding season, male golden mantella frogs aggregate to call and attract females.

3.2.5 Ethogram

Golden mantella frogs were videotaped 30 mins a day for a week prior to playback experiments; this footage was used to construct an ethogram (Table 6).

Table 6. Golden mantella frog ethogram used for behavioural analysis during playback experiments.

Behaviour	Category	Description
Jumping	Movement	Forwards whole body movement in which all four limbs briefly leave contact with the surface substrate
Crawling	Movement	Forward whole body movement in which at least two limbs retain contact with surface substrate
Calling	Social	Vocalisation, single or series of audible calls
Eating	Other	Ingestion of food
Fighting	Social	Offensive or defensive social interaction/s may include displacement from position, lunging/leaping at another individual or wrestling
Chasing	Social	The act of following another individual in close proximity
Active	Other	Stationary, no obvious activity beyond perching/sitting
Breeding	Social	Male rubbing femoral glands on the dorsum of the female
Others	Other	Other behaviour not listed
Non-visible	Other	Animal cannot be seen by the observer

All social interactions were monitored by the researchers to ensure that no frogs became injured or ill as a consequence of the playback experiments. The frogs were monitored for several weeks after the experiments and none became ill or showed any signs of distress. All experimentation was done in compliance of the relevant animal welfare laws of the country (e.g. Animal Act 1986) where conducted and followed the Association for the Study of Animal Behaviour's Guidelines for the care of animals (ASAB, 2014).

3.2.6 Behavioural analysis

All playback experiments were recorded and videos were analysed using the BORIS software (Friard & Gamba, 2016) that allows precise behavioural data to be collected. Golden mantellas' behaviour was collected before, during the playback and after using instantaneous scan sampling with 20 seconds intervals. Data on golden mantellas' behavior was quantified by summing scan samplings and the total number of each behavior recordings per playback experiment analyzed.

3.2.7 Statistical analysis

Behavioural data were tested for normality using a Shapiro-Wilk test; data did not show a normal distribution even after transformations. Generalised linear mixed models (GLMM) using the R package "MCMCglmm" (Hadfield, 2010), were used to compare the mean number of behavioural recording before, during and after the playbacks. GLMM were also used evaluate the effects of the different playback treatments on the behaviour of golden mantella frogs, replicates and tanks were used as random factors. All statistical analyses were done using R Studio (2015).

3.4 Results

Analysis of the behaviour of Chester Zoo's golden mantella prior to the playback calls showed that, from the sum of the total recordings, frogs would spend most of their time as active (25%) or non-visible (65%). During the playbacks, an increase of behaviours being displayed was observed, especially when the wild calls were played. After the 10 min playback, golden mantellas returned to the same behaviour pattern as before the playbacks (Figure 16). The GLMM showed no differences among behaviours observed before and after the playback. However, a statistical difference was found between before and during playbacks and also, during and after playbacks for all behaviours ($p<0.005$).

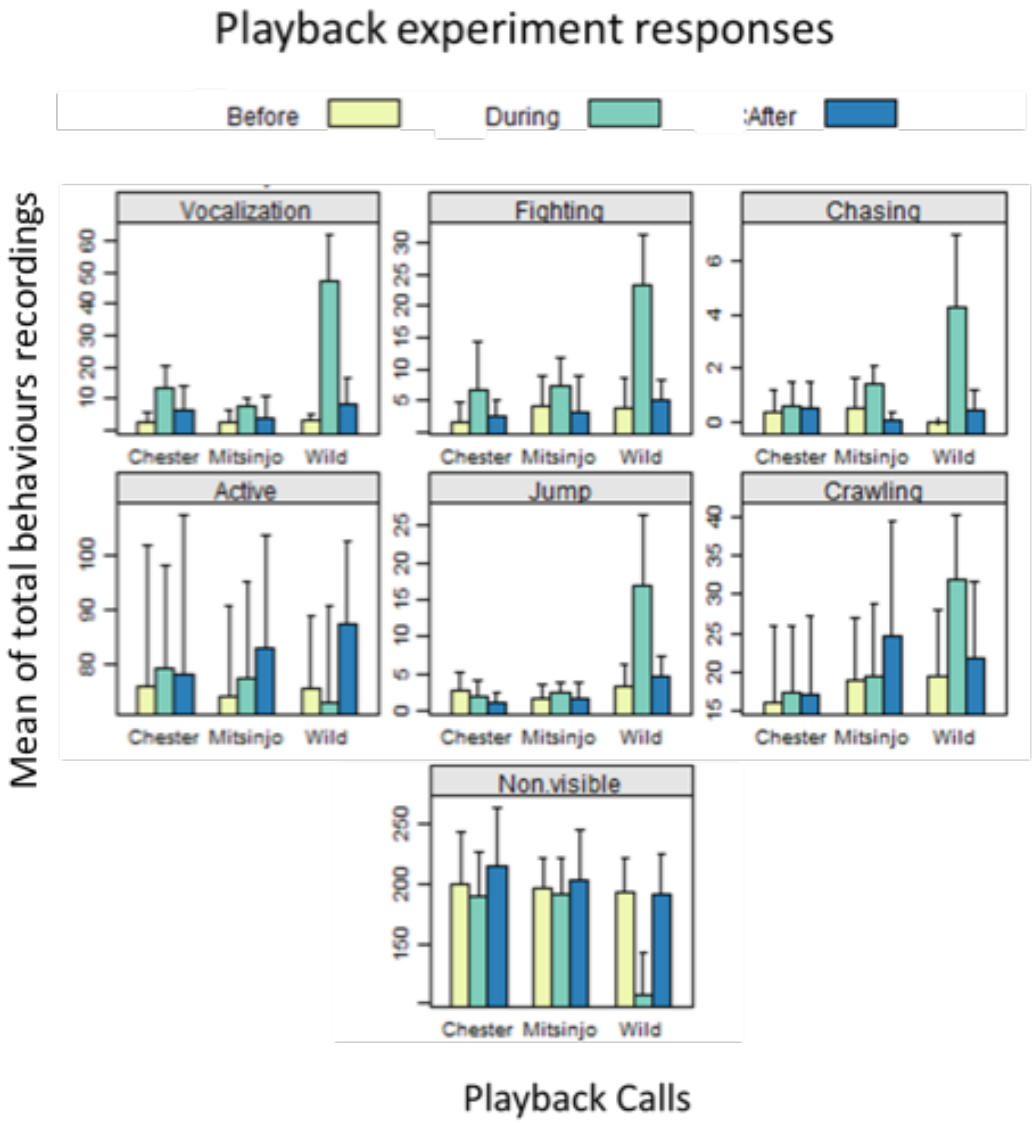

Figure 16. Mean of total behaviours recordings observed for the Golden mantella frogs before, during and after playback experiment using calls recorded from Chester Zoo, Mitsinjo Breeding Centre and wild colonies. Error bars show ± standard error of the mean

The GLMM model (see Table 7) showed that vocalisations from wild individuals lead to a significant increase ($p<0.005$) in fighting, calling, chasing, jumping and crawling behaviours, and a significant decrease ($p<0.005$) of non-visible individuals. Playback experiments using calls from Mitsinjo Breeding Centre, lead to a significant increase of

fighting, chasing, jumping and calling behaviours. Calls from Chester Zoo only resulted in an increase in calling behaviour ($p<0.005$).

Table 7. Parameter estimates for the Generalized Linear Mixed Models describing the relationship between playback treatment (call origin) and behaviour as the predictor variable for golden mantella frogs.

Behaviour	Treatment	Mean (N)	St. Deviation	l-95% CI	u-95% CI	p-value
Calling	Mangabe	19.61	±22.56	1.590	3.009	<0.001
Calling	Mitsinjo	11.05	±13.13	-0.281	1.198	0.004
Calling	Chester	8.08	±7.62	0.233	1.704	0.008
Fighting	Mangabe	10.91	±10.84	1.698	3.422	<0.001
Fighting	Mitsinjo	4.91	±5.43	0.460	2.248	0.004
Fighting	Chester	3.73	±5.44	-0.057	1.737	ns
Chasing	Mangabe	1.64	±2.55	1.325	3.390	0.001
Chasing	Mitsinjo	0.70	±0.97	0.038	2.165	0.018
Chasing	Chester	0.50	±0.92	-1.072	1.512	ns
Jumping	Mangabe	8.61	±8.63	2.056	3.591	<0.001
Jumping	Mitsinjo	2.02	±1.69	-0.019	1.599	0.050
Jumping	Chester	2.08	±2.15	-0.289	1.332	ns
Crawling	Mangabe	24.79	±10.27	0.288	1.078	<0.001
Crawling	Mitsinjo	21.52	±11.07	-0.281	0.569	ns
Crawling	Chester	17.17	±9.40	-0.354	0.460	ns
Non-visible	Mangabe	164.02	±52.81	-0.823	-0.581	<0.001
Non-visible	Mitsinjo	195.14	±32.67	-0.213	0.012	ns
Non-visible	Chester	198.79	±39.00	-0.233	-0.021	ns

3.5 Discussion

The playback experiment showed that captive golden mantellas do recognise and respond to calls from wild golden mantella frogs. Wild vocalisations created a significant increase in movement and social behaviours from captive frogs, whereas calls from captive populations did not lead to such an increase in these behaviour patterns. We also observed that calls from animals that were in captivity for more generations (more than 5 generations; i.e. Chester colony) provoked fewer responses from golden mantella frogs than calls from frogs that were in captivity for only one generation (i.e. Mitsinjo population).

The behavioural response observed during our playback experiment using wild frogs' calls, was similar to the behavioural patterns described for wild individuals during the breeding season (Edmonds et al., 2015). However, the same reaction was not observed when captive frogs were subject to playback using calls from captive frogs. A previous study has showed that captive golden mantella frogs can have their calls altered by captive conditions, animals kept in captivity for more than 5 generations had their calls significantly affected by their environment, while frogs that have been in captivity for only one generation, still possessing calls similar to wild ones (Passos, Garcia & Young, 2017).

This difference observed on wild and captive calls would explain some of the results found during the playback experiment, with calls from Mitsinjo frogs leading to a greater increase in social behaviours, while calls from Chester Zoo animals did not lead to such responses. In anurans significant information about the individual's fitness is transmitted by acoustic signals (Duellman & Trueb, 1986; Ryan, 1988) and plays an important role in sexual selection during male–male competition and female choice in many species (Marshall, Humfeld & Bee, 2003). The calls of captive frogs could be, presumably, lacking

characteristics needed to attract other individuals and elicit full reproductive behaviour, which in a reintroduction programme would have negative consequences (Sun & Narins, 2005). A low frequency of breeding between captive-bred and wild animals would also mean no improvement of the wild population's genetic diversity (Slade et al., 2014; Edmonds et al., 2015).

Maintaining wild-type behaviour such as, communication, courtship and male-male combat is relevant for successful reproduction in captive and for reintroduction programmes (Farmer, Plowman & Leaver, 2011; Schulte-Hostedde & Mastromonaco, 2015). Chester zoo's golden mantella frog captive colony, besides being in captive for over 5 generations, still has their natural breeding behaviour and can recognize wild calls (i.e. respond appropriately to wild conspecific calls). Captive breeding for reintroduction is often criticized for animals adapting to captivity and losing natural behaviour, however, the (Bloxam & Tonge, 1995; Connolly & Cree, 2008; Hunt et al., 2011) results found here shows that captive animals can maintain natural response to wild calls. Previous studies had demonstrated that wild frogs can recognize calls from captive golden mantellas (Passos, Garcia & Young, 2017). The behavioural integrity of wildlife is one of the most important aspects to conserve in captive population (Schulte-Hostedde & Mastromonaco, 2015).

Communication is essential for reproductive success in golden mantella frogs; if individuals are being bred for conservation it is of critical importance to make sure that captive animals, if released, will have the same chances of breeding as their wild counterparts. Captive breeding is growing as a conservation tool for many species (Rahbek, 1993), especially amphibians. However, it is important to fully understand the impact of captivity on a species' behaviour before releasing individuals back into the wild.

Chapter 4 –The tonic immobility test: Do wild and captive Golden Mantella frogs (*Mantella aurantiaca)* have the same response?

* This chapter has already been published at PlosOne (Passos, L.F., Garcia, G. & Young, R.J., 2017b. The tonic immobility test: Do wild and captive golden mantella frogs (*Mantella aurantiaca*) have the same response ? *PloS one*, 12(7)) See appendix

4.1 Abstract

Adaptations to captivity that reduce fitness are one of many reasons that explain the low success rate of reintroductions. One way of testing this hypothesis is to compare an important behavioural response in captive and wild members of the same species. Thanatosis is an anti-predator strategy that reduces the risk of death from predation by giving animals on last chance to escape, which is a common behavioural response in frogs. The study subjects for this investigation were captive and wild populations of *Mantella aurantiaca*. Thanatosis reaction were measured using the Tonic Immobility (TI) test, a method that consists of placing a frog on its back, restraining it in this position for a short period of time and then releasing it and measuring how much time was spent feigning death. To understand the pattern of reaction time, morphometric data were also collected as body condition can affect the duration of thanatosis. The significantly different TI times found in this study, one captive population with shorter responses, were principally an effect of body condition rather than being a result of being in captive. However, this does not mean that we can always dismiss the importance of rearing environment in terms of behavioural skills expressed.

Keywords: behavioural skills, conservation, death feigning, body condition

4.2 Introduction

Considerable difficulty has been encountered in successfully reintroducing endangered species into their natural habitats, and adaptations to captivity that reduce fitness in the wild (e.g. lack of predator recognition and appropriate response) are one of several reasons for this low success rate (Frankham, 2008). If captive animals are to be released into the wild, these issues should be addressed (Germano & Bishop, 2008). Evaluating the behavioural skills of captive bred animals could allow the selection of appropriate individuals and lead to improvements in the success rates of reintroduction programs (Reading, Miller & Shepherdson, 2013). This has been shown for different species such as black-footed ferrets (*Mustela nigripes*) (Reading, Miller & Shepherdson, 2013), Caribbean rock iguanas (*Cyclura* sp.) (Alberts, 2007) and different fish species (Griffin, Blumstein & Evans, 2000).

One of the most important responses to preserve in captive populations destined for reintroduction is the ability to detect and respond appropriately to natural predators (Kraaijeveld-Smit et al., 2006; Alberts, 2007). It is known that captivity can cause animals to lose natural responses, have insufficient fear of humans, and express abnormal behaviour (Balmford, Mace & Leader-Williams, 1996; Griffin, Blumstein & Evans, 2000; Gilligan & Frankham, 2003). These can limit the success of subsequent reintroduction attempts (Balmford, Mace & Leader-Williams, 1996; Griffin, Blumstein & Evans, 2000; Gilligan & Frankham, 2003). Captive environments are often highly predictable and without threatening stimuli; lead to important anti-predator responses being weakened or even disappearing during generations of captive breeding (Griffin, Blumstein & Evans, 2000; Kraaijeveld-Smit et al., 2006; Teixeira et al., 2007).

Tonic immobility (TI), or thanatosis, is behavioural motor inhibition and reduced responsiveness to external stimulation induced by physical restraint (Suzuki, Ikebuchi & Okanoya, 2013). Tonic Immobility has been documented as a behaviour expressed by a wide variety of species including mammals, insects, reptiles, birds, fish and amphibians (Honma, Oku & Nishida, 2006; Machado, Galdino & Sousa, 2007; Teixeira et al., 2007; Miyatake et al., 2009; Toledo, Sazima & Haddad, 2010; Fureix & Meagher, 2015). The TI response is considered as an adaptive behavioural anti-predator strategy, reducing the threat of death from predation and, thereby, increasing the chances of survival (Toledo, Sazima & Haddad, 2010). While displaying thanatosis an animal adopts a posture that gives it the appearance of being dead with which it may inhibit or divert the attack of a potential predator (Toledo, Sazima & Haddad, 2010). Toxic animals, such as golden mantella frogs, display conspicuous body coloration, and their immobile posture would often enhance the effectiveness of aposematism (Johnson & Brodie, 1975). Tonic immobility could induce the predator to loosen its hold on the prey, thereby providing a chance of escape (Toledo, Sazima & Haddad, 2010; Fureix & Meagher, 2015).

This response is associated specifically to threatening situations; the more intense the stimulus is, the longer the TI response is (Toledo, Sazima & Haddad, 2010). It is known that different factors can influence thanatosis duration such as stress levels (Morgan & Tromborg, 2007), welfare status (Fureix & Meagher, 2015), stimulus intensity (Narayan, Cockrem and Hero, 2013b), predation pressure (Narayan, Cockrem & Hero, 2013a) and environmental disturbances (Nash, Gallup & Mcclure, 1970) amongst others. Studies with frog species have demonstrated that stressful stimuli such as loud noises (*Rana pipiens;* Nash, Gallup & Mcclure, 1970), extreme temperatures (*Rana temporaria*; Dabrowska and Manikowski, 1982) or the sight of predators (*Platymantis vitiana;* Narayan, Cockrem & Hero, 2013b) can affect TI response duration of captive animals.

It is crucial to conserve the behavioural integrity of captive wildlife, particularly if animals are to be used for conservation efforts including reintroductions (Schulte-Hostedde & Mastromonaco, 2015; Young, 2003). The aim of this study was to compare tonic immobility responses of wild and captive golden mantella frogs (*Mantella aurantiaca)*, thereby assessing the effects of captivity on this survival strategy. As death feigning is a natural defensive response (Honma, Oku & Nishida, 2006; Toledo, Sazima & Haddad, 2010; Narayan, Cockrem & Hero, 2013b) it was predicted that wild frogs will have a longer TI response since these individuals are expected to be more experienced in expressing defensive behaviours due to the threats in their habitat. Captive bred animals can be naive to the threat of predation and, therefore, might be unable to generate adequate physiological and behavioural responses to a threatening stimulus (Narayan, Cockrem & Hero, 2013b). Tonic immobility is also associated with fear (Narayan, Cockrem & Hero, 2013b), since captive frogs are also habituated to handling and human interaction (e.g. during cleaning and feeding routines): a human interaction should not trigger such a fear response (Morgan & Tromborg, 2007).

4.3 Methodology

4.3.1 Study subjects

The study subject was the golden mantella frog, for more details please see section 1.8.

4.3.2 Study sites

Mangabe Area: Data sampling for this study was done in the Moramanga region. The data from wild frogs (N = 90) at Mangabe were obtained during October 2014 and again in February 2015 (both during breeding season). For more detail, please see section 1.9.

Ambatovy Mining Site: During this study, animals from the Conservation Zone and animals that were translocated to Receptor ponds were sampled. Ambatovy population (N = 30) was sampled in March 2016. For more detail, please see section 1.9

Chester Zoo, UK: Animals are fed different live invertebrates with diet supplementation. The Chester Zoo population (N = 30) was sampled in March 2016. For more detail, please see section 1.9

Mitsinjo Association Captive Breeding Centre: During this project, only data from the founders' offspring (F1) were collected. The data from the captive frogs from the Mitsinjo captive breeding centre (N = 20) were obtained in February 2015.

4.3.3 TI Test

Thanatosis reaction was measured using the Tonic Immobility (TI) test, a standardised method that consistently and reliably induces TI (Suzuki, Ikebuchi & Okanoya, 2013; Fureix & Meagher, 2015). Frogs were caught and immediately subjected to the TI test (within 3 s). Each individual was placed on its back in the palm of the experimenter's hand and restrained in that position for 10 s using gentle pressure on its belly from the experimenter's thumb, and then released (Figure 17). If a frog moved 3 s after release, then it was considered that TI had not been induced. In this case, the restraint was repeated up to three times. If TI was not induced after 3 attempts, a score of 0 s was given. Conversely, if frogs did not show any movement after 5 min, the test was terminated and a maximum score of 300 s was given for tonic immobility duration. Animals were always handled by the same researcher. Tonic immobility can be affected by ambient temperature (Dabrowska & Manikowski, 1982; Miyatake et al., 2009), Chester Zoo facilities are kept in a temperature controlled environment to mimic Madagascar climate conditions. Mitsinjo facilities' temperature is allowed to fluctuate with the climate outside since the captive population was maintained within the native range of the species (Edmonds et al., 2015). For this reason temperature was not used as a possible source of variation (i.e. factor).

Figure 17. Golden mantella frog during tonic immobility response (Photo by: Luiza Passos*)*

4.3.4 Body Condition Index

Body condition index (BCI) was assessed using the Scaled Mass Index proposed by Peig & Green (2009). This method is independent of size and can be used for comparison between different populations; these characteristics potentially make it superior to the traditional residual indices and, reportedly it has worked well in amphibian studies (Maccracken & Stebbings, 2012; Michaels, Antwis & Preziosi, 2014). The scaled mass index of condition (M_i) was calculated as follows:

$$M_i = M * [SVL_0/SVL]^{bSMA}$$

where M and SVL are the mass and Snout-vent length of the individual, SVL_0 is the arithmetic mean SVL of the population, and bSMA is the standardized major axis slope from the regression of ln M on ln SVL for the population (Peig & Green, 2009). Each individual was measured (±0.01mm) for SVL using a digital calliper (Lujii 150 mm, Omiky) and body mass (Figure 18.) was obtained using a precision scale (accurate to 0.01g, Smart Weigh ACC200 AccuStar).

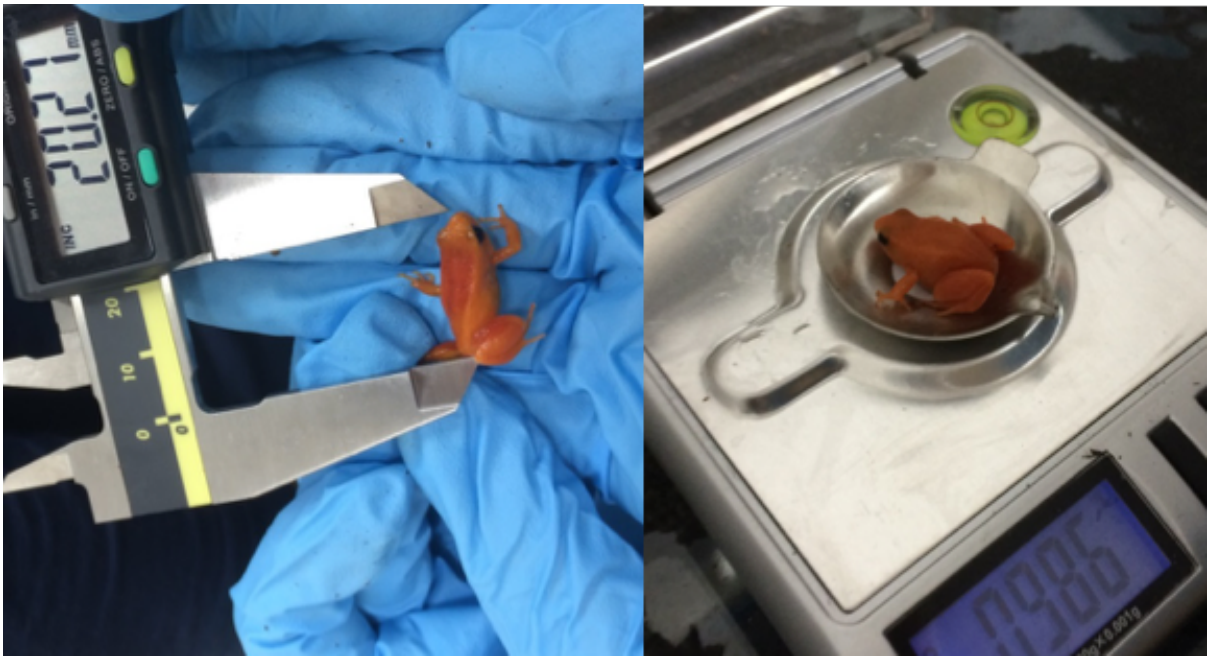

Figure 18. Golden mantella frog's morphometric measurements (Photo by Gerardo Garcia).

4.3.5 Data Analysis

TI responses and BCI data were confirmed to have a normal distribution using the Shapiro-Wilk normality test. There were no statistical differences between BCI and TI responses between the two sample periods in Mangabe, and between the two populations from Chester Zoo, for this reason, data were analysed together. No statistical difference was observed between male and female responses, thus data was analysed together. TI responses and BCI were compared using ANOVA tests. A Pearson correlation was used to analyse BCI and TI responses. Statistical analyses were done using R Studio (RStudio team, 2015).

4.4 Results

There was no significant difference in TI responses among groups (wild and captive) ($F_{1,199}$=1.90, p=0.17), but there was a significant difference between populations (Chester Zoo, Mitsinjo, Ambatovy and both sampling periods for Mangabe) ($F_{4,199}$=12.23, p<0.001). The Tukey *post-hoc* analyses showed that the golden mantella frog population kept at Mitsinjo Breeding Centre had a significantly (p<0.01) shorter duration TI response when compared to all other groups (Table 8) and no other significant differences were detected.

Table 8. Tonic immobility test results for different wild and captive populations of golden mantella frogs.

Population	Group	N	Max (secs)	Min (secs)	Mean (secs)	St. Dev (secs)
Mangabe	Wild	90	180	0	78.54	47.40
Ambatovy - Receptor	Wild	30	147	0	81.00	67.00
Ambatovy - Conservation	Wild	30	180	0	71.31	59.06
Mitsinjo Breeding Centre	Captive	20	40	0	10.05	13.72
Chester Zoo	Captive	30	136	30	83.63	29.99

After obtaining a body condition index for all individuals (Table 9), groups (wild x captive) were compared using a one-way ANOVA test ($F_{1,199}$=8.278, p= ns). The test showed that there was no significant difference between groups. When data from the populations were compared and a significant difference between populations ($F_{4,199}$= 9.289, $p<0.001$). The Tukey *post-hoc* analyses confirmed that animals from Mitsinjo were significantly different from all other groups with a much lower body condition.

Table 9. Body Condition Index Score results for different wild and captive populations of golden mantella frogs.

Population	Group	N	Max	Min	Mean	St. deviation
Mangabe	Wild	90	1.54	0.42	0.89	0.16
Ambatovy - Receptor	Wild	30	2.29	0.56	0.88	0.40
Ambatovy - Conservation	Wild	30	1.01	0.49	0.87	0.11
Mitsinjo Breeding Centre	Captive	20	1.28	0.39	0.67	0.19
Chester Zoo	Captive	30	1.12	0.40	0.91	0.32

A significant positive correlation was found between TI responses and body condition index scores when all data were compared using a Pearson correlation test ($r=0.22$, $N= 200$, $p<0.05$; observation: 4 outliers removed ($r=0.33$, $N= 196$, $p<0.001$), which had very large standardised residuals) and when each population was analysed separately (Table 10, Figure 19). Animals with better body condition had longer responses.

Table 10. Pearson correlation results for relationship between tonic immobility response (duration) and body condition index for different golden mantella frog populations

Population	r	N	p-Value
Mangabe	0.26	90	<0.05
Ambatovy - Receptor	0.37	29	<0.04
Ambatovy -Conservation	0.32	29	<0.02
Mitsinjo Breeding Centre	0.51	19	<0.05
Chester Zoo	0.38	29	<0.04

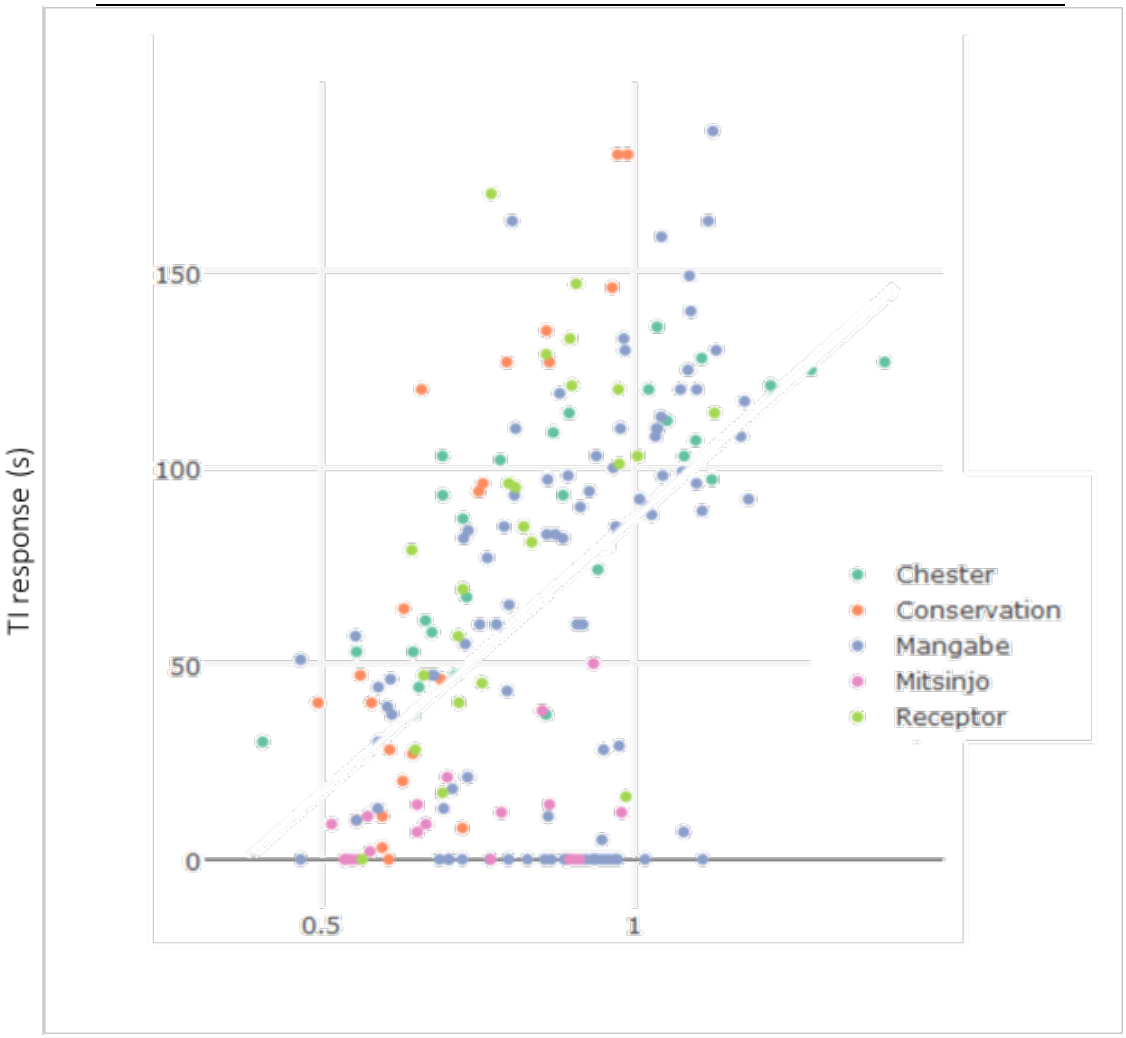

Figure 19. Scatter plot of body condition index (BCI) and tonic immobility (TI) response (s) of different populations of golden mantella frogs.

4.5 Discussion

In this study we showed that wild populations of golden mantella frogs and those kept at Chester Zoo had similar TI response durations, whereas animals kept at Mitsinjo breeding centre had a significantly shorter TI duration. These results suggest that captivity is not the only factor involved in the shorter durations observed in one of the captive colonies. Animals from Chester Zoo, which have been in captivity for many more generations, still presented the same response as the wild populations. On the other hand, frogs kept at Mitsinjo Breeding Centre after the first generation in captivity presented a shorter response when compared to wild animals. This is true even when compared to the wild population from where their parental generation were collected, which also discounts the results being due to some natural variation between populations.

During this study, there was also a significant difference in the body condition of animals between the populations. Body condition is a valuable index that can be assessed using reliable, non-invasive techniques, and it can identify the health condition of a population before any deleterious effects can be observed (Maccracken & Stebbings, 2012). The data collected from wild and captive *M. aurantiaca* showed that the individuals kept at the Mitsinjo breeding centre had a much lower body condition index than any other group. Again, this cannot be generalized as a consequence of captivity, since frogs from Chester Zoo present no statistical difference on BCI when compared to the wild populations. This result could be used to infer that animals at Mitsinjo are not in ideal health condition when compared with other analysed populations.

Lower body condition could be a result of different factors such as diet, reproductive stage and age (Labocha, Schutz & Hayes, 2014). Both captive colonies receive a diet of variety of live invertebrates, but Chester Zoo's colony also received a diet supplementation. There is a lack of knowledge concerning the nutritional necessities and absorption efficiency

of amphibians; however, studies have demonstrated that diet supplementation can have a positive impact on frog body condition and general health (Livingston et al., 2014). This lack of vitamin and mineral supplementation could be causing frogs from Mitsinjo to have a lower body condition.

There is also a reported relationship between weight-loss and stress in captive individuals (Morgan & Tromborg, 2007; Labocha, Schutz & Hayes, 2014). Captivity can present many sources of stress, possibly the greatest stressors are those over which the animal has no control and from which they cannot escape, such as a poor diet, inadequate habitat and restricted movement (Morgan & Tromborg, 2007). Chronic stress may be indicated by a wide range of physiological responses including inhibited growth rate (Chrousos, 1997; Tsigos & Chrousos, 1995), reduced body weight (Bartolomucci et al., 2004; Konkle et al., 2003), and reduced food intake (Schumann et al., 2014). Persistent exposure to continuous stressors can have many deleterious consequences for captive animals putting the long-term health of captive animals at risk (Broom & Johnson, 1993; Chrousos, 1997; Sapolsky, 1996; Wendelaar-bonga, 1997; Young, 2003). Environmental factors, such as providing the correct UV light standards, could be involved in maintaining the healthy state of frogs kept in captivity (Antwis & Browne, 2009; Livingston et al., 2014; Tapley et al., 2015). The lack of UV light provision for the Mitsinjo colony could, also, be involved at the low body condition.

The positive correlation between TI response and BCI showed that body condition was an important factor in the duration of the tonic immobility response; individuals with lower body condition had shorter responses independent of origin. Even though a correlation was found it is important to state that it was a weak correlation. Possibly other factors are involved in the TI responses. The results found here showed that husbandry differences, and not just being in captivity per se, had an impact on the health conditions of frogs and as a consequence affected their behavioural responses.

TI response is an acute stress response to a short-term elevation of corticosterone levels, as has already been demonstrated in experiments using Fijian ground frogs (*Platymantis vitiana)* (Narayan, Cockrem & Hero, 2013b). A short-term elevation of stress hormones could be caused by a predator attack or the simulation of one (Tonic immobility test). A short-term increase in the corticosterone levels can promote key changes in the behaviour and physiology that enables individuals to cope with stress (Narayan, Cockrem & Hero, 2013a): an acute stress response. Some of the key behaviours affected by corticosterone in amphibians are defensive behaviours such as tonic immobility (Narayan, Cockrem & Hero, 2013b). However, if frogs from Mitsinjo were already experiencing chronic levels of stress due to a poor diet and environment, it is possible that their acute stress responses could be blunted (Tapley et al., 2015), such as TI responses.

Body condition index can be used to assess the chronic levels of stress of captive animals (Broom & Johnson, 1993), while TI response could be an alternative technique to asse acute stress responses on captive individuals. The stress response is not inherently detrimental, but rather, is a complex and essential negative-feedback process (Hing et al., 2016). The capacity to cope with threatening (acute stress) situations is a vital ability to survival in the wild (Livingston et al., 2014). Predation, competition and other stressful events are part of the routine in the wild habitats.

Captive environments are different from the wild and can impose different selection pressures or relaxed selection pressures leading to adaptation to captivity and, consequently, affecting behaviour including anti-predators responses (Burghardt, 2013; Frankham, 2008; Gilligan & Frankham, 2003). The importance of maintaining the behavioural integrity of zoo populations, especially those that are used for conservation efforts including reintroductions is critical for the conservation of biodiversity (Dabrowska & Manikowski, 1982). Amphibians have long been neglected in research into animal welfare and behavioural

problems related to captivity; this is clear in the historic lack of enriched captive environments to encourage natural behaviour and psychological well-being (Burghardt, 2013).

A biosecurity facility for the conservation of amphibians on site is a very important step for the future of many different species (Burghardt, 2013). However, maintaining the necessary standards to keep animals fit for reintroductions is still a challenge. The husbandry differences, provision of UV light and diet supplementation, found between Chester Zoo and Mitsinjo reflect the availability of equipment and diet supplements in each country. Reintroductions are costly and time consuming; therefore, to make the best use of resources available it is important to screen individuals that are destined for reintroduction.

Chapter 5– How does captivity affect skin colour reflectance of golden mantella frogs?

5.1 Abstract

Colouration is an important trait for social communication in amphibians, being used in intra- and intersexual signaling to express information about individual body condition and health state, amongst other things. The striking colour pattern exhibited by some anuran species are also used in "aposematic" signals to advertise unpalatability to predators. The aim of this study was to if the captive environment has affected the colour of golden mantella frogs by comparing different captive reared frogs with wild conspecifics. A USB-2000 portable diode-array spectrometer and a xenon strobe light source were used to perform spectrophotometric measurements on captive and wild frogs. Hue, chroma and brightness of skin colour were analyzed as well as body condition using the scaled mass index. Analyses showed variation among populations but significant differences were only found between captive and wild populations. Generalized linear mixed models were used to evaluate the effects of body condition on colour variation and showed that animals with lower body condition from one captive population, Mitsinjo breeding centre, had significantly different colouration from their wild counterparts. Importantly, one captive population was not greatly different in colouration from their wild counterparts – demonstrating that this problem is not inevitable in captivity. These results have important implications for reintroduction programmes.

Key-words: amphibians, body condition, colouration, conservation

5.2 Introduction

One central question in evolutionary biology is understanding the role played by colouration and colour vision in species communication (Maia et al., 2013). Colouration has an essential influence on many different ecological processes such as thermoregulation, aposematism, mating and communication. For these reasons, there are many evolutionary and ecological studies focusing on understanding and quantifying animal colour variation (Chittka & Menzel, 1992; Endler, 1993; Forsman et al., 2002; Lanuza, Carazo & Font, 2014; Robertson & Rosenblum, 2009). However, subjectivity is a problem when working with colouration (Endler, 1990), colour is not a quantitative characteristic of an object, but part of a sensorial experience that varies greatly according to the species visual system (Maia et al., 2013).

Colouration can be used in intra- and intersexual signalling to convey information about individual quality such as body condition, fighting ability, territory quality, parental care, good genes, parasite resistance and immunocompetence (Bradbury & Vehrencamp, 2011; Crothers, Gering & Cummings, 2011; Mann & Cummings, 2008; Umbers et al., 2016). The remarkable colour patterns displayed by many anuran species (Hoffman & Blouin, 2000) are part of their defence strategies; for example, announcing the individual's unpalatability (i.e. a warning signal to predators about its chemical protection) (Hegna, Saporito & Donnelly, 2013; Maan & Cummings, 2012). This is a widespread strategy throughout the animal kingdom known as aposematic colouration (Ruxton & Sherrat, 2004). The fact that predators can be warned at a distance about a prey's toxicity reduces risky encounters with predators and also diminishes the cost of otherwise dangerous behaviours, such as foraging and sexual displays (Dugas et al., 2015).

Divergent antipredator strategies such as aposematism not only require integration of physiology, morphology and behaviour; they also alter the way selection acts on other suites

of traits (Stankowich & Blumstein, 2005). It is expected that, in some scenarios, higher levels of toxicity should be correlated with a more striking skin colouration, with "nastier" animals "shouting loudest" (Maan & Cummings, 2012; Speed & Ruxton, 2007). This is because the greater risk of detection and attack for highly conspicuous prey can be compensated for by the stronger predator deterrence induced by high toxicity (Darst, Cummings & Cannatella, 2006). A positive relationship may also emerge from physiological or energetic trade-offs between the two traits (Blount et al., 2012).

Amphibian skin colouration and associated patterns are mostly the result of two cell types: melanophores, which contain melanin, and chromatophores, which contain other coloured pigments (Hoffman & Blouin, 2000, Umbers et al., 2016). Chromatophores are divided into two main categories: non-reflecting that contained yellow and red pigments including carotenoids, and reflecting ones which contain pigments called iridophores (Hoffman & Blouin, 2000; Brenes-Soto & Dierenfeld, 2014).These cell types work together to produce the colours found in anuran skin. Pigment accumulation is also linked to the frog's diet (Brenes-Soto & Dierenfeld, 2014; Ogilvy, Preziosi & Fidgett, 2012).

It is fairly common to observe amphibians kept in captivity displaying a faded colouration in comparison to their wild counterparts (Brenes-Soto & Dierenfeld, 2014; Ogilvy, Preziosi & Fidgett, 2012). Mimicking diet and environmental condition in captivity is one of the major challenges faced while keeping frogs in captivity (Livingston et al., 2014), this can directly affect amphibian skin pigmentation (Brenes-Soto & Dierenfeld, 2014). Alteration of colouration may also affect potential recognition of breeding partners, perception of fitness, and could also have effects on health and reproductive output (Brenes-Soto & Dierenfeld, 2014). If animals are being bred for conservation purposes and a reintroduction is a future goal, these issues are of major concern. Therefore, the aim of this

study was to investigate and quantify how the captive environment affects the colour of golden mantella frogs by comparing zoo reared frogs with wild conspecifics.

5.3 Methodology

5.3.1 Study subject

The study subject was the golden mantella frog. This is an ideal species to test the effects of captivity on colouration because the species is naturally only one uniform colour (i.e., orange). For more details please see section 1.8.

5.3.2 Study sites

Mangabe area (Madagascar wild): Data sampling (15 males and 15 females) for this study was done in a protected area of the Moramanga region. For more details please see section 1.9.

Ambatovy Mining Site (Madagascar wild): During this study animals from the Conservation zone (15 males and 15 females) and animals that were translocated to Receptor ponds (15 males and 15 females) were sampled. For more details please see section 1.9.

Chester Zoo (UK): We sampled 8 males and 8 females from the public on-show frogs and the same number from the off-show frogs, that have been in captive for over seven generations. For more details please see section 1.9.

Mitsinjo Association Captive Breeding Centre (Madagascar captive): We sampled 8 males and 8 females founder frogs and the same number from the F1 frogs. For more details please see section 1.9.

5.3.3 Spectrophotometric measurements

We used a USB-2000 portable diode-array spectrometer and a PX-2 xenon strobe light source (both from Ocean Optics, Dunedin, USA), with the probe positioned at an angle of 90° to the animal being sampled, to perform spectrophotometric measurements. To

exclude ambient light and standardize measuring distance, a cylindrical plastic tube was mounted on the fibre optic probe. The equipment permitted that the spectral analyses were conducted in the 300 and 700 nm range. Spectral reflectance measurements were always taken of each individual from the dorsum, three measurements per frog (Figure 20). Colour measurements should sample the most visible surfaces to obtain a representative sample (within an individual) of the spectral shape of the entire body. Summary variables for the colour measurements were calculated. Spectralon white standard measurements were taken between each individual to account for lamp drift. This methodology was based in previous studies measuring colour variation in different species (Siddiqi et al., 2004; Crothers, Gering & Cummings, 2011; Maan & Cummings, 2012).

Figure 20. Spectral reflectance measurements being taken from golden mantella frogs at Mangabe (Photo by: Gerardo Garcia)

5.3.4 Colour analyses

Colour may be described by using four essential parameters: Hue, Chroma, and Brightness (i.e. Intensity) and these variables were analysed due to them being customarily used in studies of animal coloration, thereby facilitating comparisons between studies. Brightness (Qt) may be defined as the total intensity of light (Endler, 1990), Qt was calculated by summing the percentage reflectance (R) across the entire spectrum (R300 and R700).

Hue is the everyday meaning of colour, for example, violet, blue, orange, green (Endler, 1990); In general, the hue of a spectrum is a function of its shape. Hue is correlated with the wavelength of the maximum slope, as well as the sign of the slope (Endler, 1990). It is the wavelength within the visible-light spectrum at which the energy output from a source is greatest (Maia et al., 2013). Hue (nm) was measured as the wavelength of maximum reflectance.

Chroma is a measure of the 'purity' or 'saturation' of a colour, and is a function of how rapidly intensity changes with wavelength (Endler, 1990). Chroma was calculated as relative medium wavelength chroma (MC, calculated as (Rmax – Rmin)/Qt). Brightness, Hue and chroma differences between populations were analysed with a mixed model with origin (wild or captive) as fixed factors and populations as random factors. Carotenoid chroma can only be used when the colour of the surface is clearly due to carotenoid pigmentation (Maia *et al.*, 2013).

Data were analysed using the Pavo (Maia et al., 2013) package from R Studio (2015). The data from each population were plotted on the same graph to confirm standardization of

sampling, no error from the sampling was found (Figure 21). Data from different populations were compared based on colour distance and colorimetric variables.

The colour distance analyses applies the visual models of any chosen species to calculate colour distances based on visual system sensitivity, it allows to know if an animal species would be able to perceive differences between colours (Maia et al., 2013). Four different visual systems, human, a snake (Boidae, Bowmaker, 1998), a Scincidae lizard (New et al., 2012) and a diurnal poison frog (*D. pumilio,* Siddiq*i* et al*.,* 2004) were to test if the differences between samples were distinguishable. Calculations are done using Just Noticeable Distance units (JND) (Wen, 2012) to quantify the presence of differences between groups, when JND=1, the spectral pair is barely discriminable under ideal conditions, and as JND becomes greater, discrimination can be made more rapidly and under increasingly unfavourable viewing conditions (Siddiqi et al., *2004*).

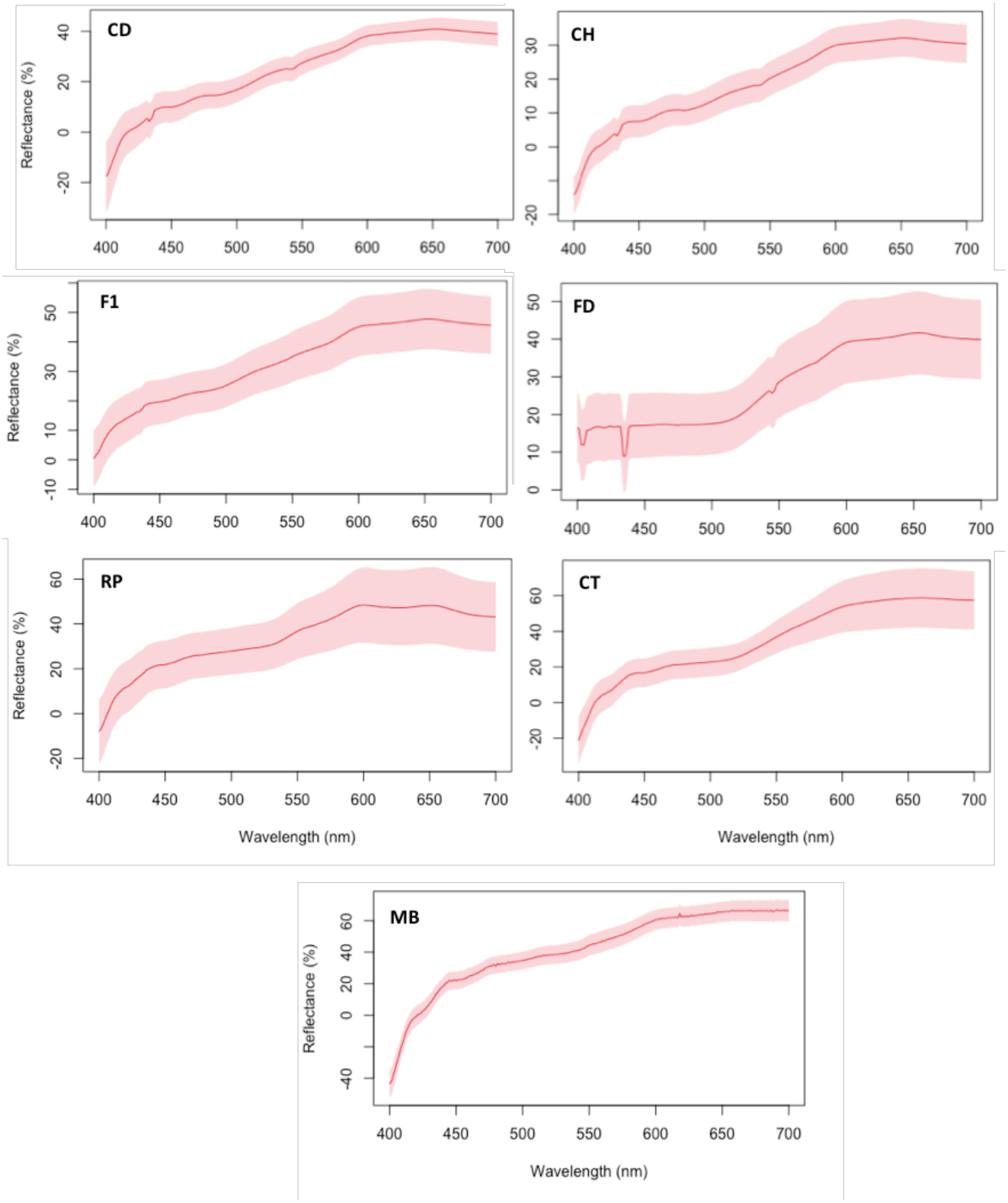

Figure 21. Mean (dark line) ±SE (lighter colour) spectra reflectance of each golden mantella frog population. CD = Chester Zoo public display, CH = Chester Zoo off-show, F1 = First offspring Mitsijo, FD= Founders, RP = Receptor ponds, CT = Conservation zone, MB = Mangabe.

5.4 Results

The colorimetric variables analysis showed no differences between wild and captive animals for brightness, but significant differences ($p<0.0001$) for hue, chroma and carotenoid chroma were found (Figure 22).

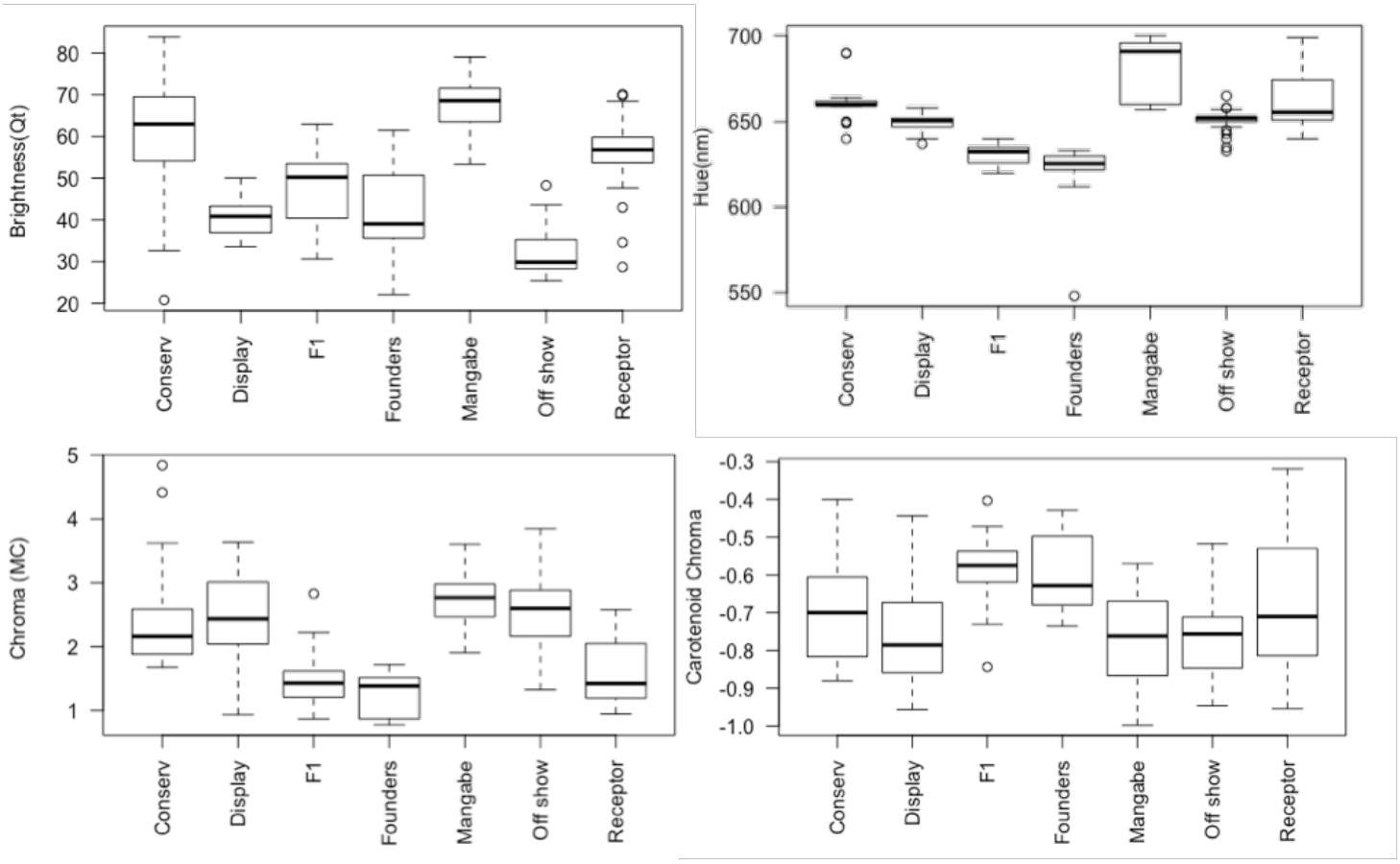

Figure 22. Colorimetric variables for skin colouration analysis for different wild and captive populations of golden mantella frogs. Thick line show median, whiskers show maximum and minimum values and box encompasses upper and lower quartile.

The colour distance analyses (Table 11) shows that the difference between the skin coloration of frogs kept at Mitsinjo and wild individuals would be highly noticeable for all visual systems tested. This difference would also be noticeable when comparing the skin colouration of Chester zoo and Mitisnjo populations. The individuals from Chester Zoo and the animals from Mangabe have a low or non-detectable difference in the colour distance analyses.

Table 11. Colour distance analysis using human, snake, lizard and frogs' visual systems showing differences between different skin colouration of golden mantella frog populations.

Groups	Colour distance (JND units)*			
	Human	Snake	Lizard	Frog
Chester Display^{C} – Chester off^{C}	0.92	0.52	0.35	0.35
Chester Display^{C} – F1 (Mitisnjo) ^{C}	9.52	5.43	6.55	5.09
Chester Display^{C} – Founders (Mitisnjo) ^{C}	12.09	3.93	2.87	3.09
Chester Display^{C} – Mangabe^{W}	1.95	1.06	1.05	1.32
Chester Display^{C} – Conservation zone^{W}	4.46	2.13	2.48	3.22
Chester Display^{C} – Receptor pond^{W}	3.10	1.69	2.51	1.12
Chester off^{C} –F1 (Mitisnjo)^{C}	**10.44**	4.95	**6.57**	4.73
Chester off^{C} –Founders (Mitisnjo)^{C}	**10.00**	3.72	5.61	4.88
Chester off^{C} - Mangabe^{W}	1.65	1.37	1.41	1.67
Chester off^{C} – Conservation zone^{W}	3.36	3.61	1.15	2.87
Chester off^{C} – Receptor pond^{W}	3.02	1.19	2.53	0.76
F1(Mitisnjo)^{C} – Founders (Mitisnjo)^{C}	2.62	2.38	2.44	3.09
F1(Mitisnjo)^{C} - Mangabe^{W}	**9.32**	**6.21**	**6.33**	5.41
F1(Mitisnjo)^{C} – Conservation Zone^{W}	5.10	4.75	**6.28**	2.13
F1(Mitisnjo)^{C} – Receptor pond^{W}	**8.42**	4.33	5.04	3.05
Founders (Mitisnjo)^{C} - Mangabe^{W}	**11.91**	5.62	5.76	3.82
Founders (Mitisnjo)^{C} – Conservation zone^{W}	**7.64**	4.71	4.60	3.27
Founders (Mitisnjo)^{C} – Receptor pond^{W}	**8.04**	4.28	2.45	4.03
Mangabe^{W} – Conservation zone^{W}	1.32	2.78	2.52	3.55
Mangabe^{W} – Receptor pond^{W}	2.90	2.39	1.02	2.44
Conservation zone^{W} – Receptor pond^{W}	2.70	2.47	1.61	2.10

*Just Noticeable Difference units reference values: 0-1 not detectable; 1-2 Low; 2-3 Medium; 3-4 High; 4-5 Very high; >6 Extremely high, values in bold. ^{W} = wild population; ^{C} = captive population.

UV light levels were compared using an ANOVA test, and no statistical difference was found between the measurements taken at Chester Zoo (2.03±0.4) and in the natural habitat (1.03±0.29) ($F_{2,14}$= 10.23, p<0.001**)**. Mitsinjo's data were not included in this analysis due to the fact the animals kept at Mitsinjo Breeding Centre had no UV lights.

Body condition (Figure 23) analyses showed a significant difference between the groups ($F_{7,153}$=7.109, p<0.001). The *post-hoc* analyses confirmed a significant difference (p<0.0001) between the Mitsinjo group and all other groups. Frogs kept at Mitsinjo had a significantly lower body condition.

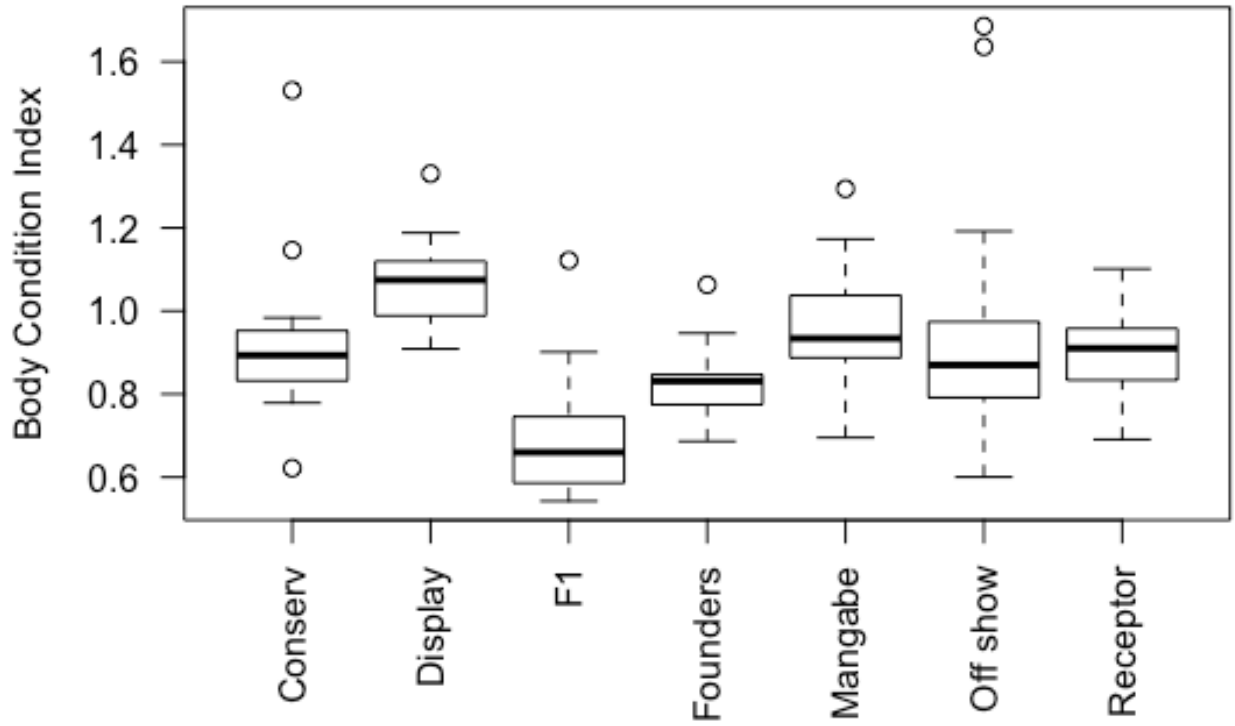

Figure 23. Body condition index scores for different wild and captive populations of golden mantella frogs. Thick line show median, error bars show max and min values and box encompasses upper and lower quartile.

Generalized linear mixed models were used to evaluate the effects of body condition on the chroma, carotenoid chroma and hue variation (see Table 12). Location was included as a random factor (chroma: variance 0.38, St. Dev. ± 0.62, hue: variance 118.13, St. Dev. ± 10.86). The selected model with an AIC of 1195.1 for chroma and AIC of 332.80 for Hue, showed that body condition had a strong impact on both chroma ($F_{1,2}$=7.17, $p<0.001$) and Hue ($F_{1,2}$=25.83, $p<0.001$) animals with lower body condition having a more affected colouration but it should be noted that this was confounded with the group that had no UV light supplied.

Table 12. Parameter estimates for the Generalized Linear Mixed Models describing the relationship between the body condition (SMI) and colorimetric variables as the predictor variable

Colourimetric variable	Fixed effects	Estimate	Std. Error	t value
Chroma	Intercept	2.23	0.35	6.21
Chroma	SMI	-0.23	0.24	-0.952
Hue	Intercept	534.72	5.74	93.08
Hue	SMI	-3.70	3.29	-1.12

5.5 Discussion

In this study, we showed that different populations of golden mantella frogs had differences in some aspects of their skin colouration, but that significant differences were only found between captive and wild frogs. In general, wild frogs were brighter, more colourful and were a different shade of orange in comparison to captive frogs, especially those from the captive populations in Madagascar (Figure 24). A relationship between lower body condition and duller colouration was observed, but this was confounded with UV light provisioning. Alteration of pigmentation could affect potential recognition of breeding partners, perception of fitness, and could thus have an indirect effect on health and

Figure 24. Examples of skin colouration from the three groups of golden mantellas. A) Wild individuals; B) Chester Zoo individuals; C) Mitisinjo individuals (*Photos by: Luiza Passos and Gerardo Garcia)*.

reproductive output (Crothers, Gering & Cummings, 2011; Ogilvy, Preziosi & Fidgett, 2012; Rojas, 2016)

The hue comparison results showed that the golden mantella frogs' skin coloration has been affected by captivity with a significant difference when compared to wild conspecifics. The observed difference on the skin colouration was quantified by the colour distance analyses that shows how a particular species (visual system chosen for analysis) would perceive the colour difference between the studied groups. The results showed that, even though, there were significant differences between how skin colouration of different populations would be perceived, most of them were low or non-detectable, except for the Mitsinjo colony, both founders and F1 presented a colouration that differed significantly from their wild counterparts. This shows that the change in the skin coloration is not a generalized effect of captivity since frogs kept at Chester Zoo did not display such a dramatic change.

A husbandry aspect that could be associated with the colour differentiation of captive frogs is the provision, or not, of UV lights. Studies on the effect of UV lights on the development of captive frogs has already shown negative consequences on the growth and skin colouration of amphibians kept with low levels of UV (Antwis & Browne, 2009; Michaels, Antwis & Preziosi, 2014). Animals kept with low levels of UV took longer to obtain full coloration or showed a pallid coloration (Michaels, Antwis & Preziosi, 2014). Golden mantella frogs kept at Mitsinjo breeding centre are not provided with additional UV light in their enclosures, both groups kept at Chester Zoo receive additional UV light and wild frogs are under the influence of natural UV in the environment. Keeping amphibians in captivity without any UV radiation could potentially have deleterious effects on the health condition and skin colorations of these individuals (Peters et al., 2004; Antwis & Browne, 2009).

The data collected from wild and captive *M. aurantiaca* showed that the individuals kept at the Mitsinjo breeding centre had a much lower body condition that any other group. Body condition is a result of many variables, nutritional requirements, stress levels and abiotic variables. Environmental factors, such as providing the correct UV light standards, could be involved in maintaining the healthy state of frogs kept in captivity (Antwis & Browne, 2009; Michaels, Antwis & Preziosi, 2014; Tapley et al., 2015). There is still a deficiency of knowledge regarding the importance of UV light for amphibian metabolism and correct levels to provide in captivity (Michaels, Antwis & Preziosi, 2014; Tapley et al., 2015), but recent studies are showing the negative effects of not providing UV for captive frogs (Antwis & Browne, 2009; Michaels, Antwis & Preziosi, 2014; Tapley et al., 2015).

The carotenoid based chroma analysis showed a significant difference between wild and captive populations. Amphibians use carotenoids for skin pigmentation, and because carotenoids are only obtainable through the diet, colour degradation could result from limited carotenoid availability in captive diets (Ogilvy, Preziosi & Fidgett, 2012, Umbers et al., 2016).Replicating diverse diets in captivity creates a range of challenges including issues of environment, economics and practicality of insect husbandry (the main food item) (Livingston et al., 2014, Umbers et al., 2016). Carotenoids have a variety of functions in immune function, reproduction, exercise performance, and coloration, and can directly influence fitness. For example, carotenoid- rich diets have been shown to improve the escape performance southern corroboree frogs (Silla, McInerney & Byrne, 2016), and increase in the number of offspring that successfully metamorphose in strawberry poison frogs (*Oophaga pumilio*) (Dugas, Yeager & Richards-Zawacki, 2013). Lower levels of carotenoid availability on diet may affect potential recognition of breeding partners, perception of fitness, and have an indirect effect on health and reproductive output (Crothers, Gering & Cummings, 2011; Ogilvy, Preziosi & Fidgett, 2012, Umbers et al., 2016)

A relationship between body condition and loss of skin coloration was detected; animals with lower body condition also had a greater difference in skin colouration according to the Colour Distance Analyses. Body condition is a consequence of different factors, such as diet, diseases and stress levels (MacCracken & Stebbings, 2012), for example, and any of those factors could also be associated with the change on the skin colouration of frogs kept at Mitsinjo breeding centre.

The colour distance analyses done using humans visual system (Bowmaker, 2015) demonstrated that keepers would be able to detect the different colouration on the animals they keep. This could be used as a measurement to selected animals with greater similarities with the wild populations for reintroduction purposes. Colour charts are commonly used to evaluated colour scores of animals in zoos (Brenes-Soto & Dierenfeld, 2014), although this is a qualitative measurement, a colouration chart species-specific, could be produced and used as a selective tool for reintroduction along side other tools, such as a health screening.

The colour distance analyses using the spectral sensitivity of a diurnal frog have shown that frogs would be able to detect colouration differences. Diurnal species of amphibians, such as the golden mantellas, use visual signals as an important part of their courtship and mate selection (Maan et al., 2004; Bowmaker, 2015). For example, females of different taxa prefer to mate with more colourful or brighter individuals (Bajer et al., 2010; Gomes et al., 2009; Maan & Cummings, 2008; Ogilvy, Preziosi & Fidgett, 2012). Releasing animals with different skin coloration could, potentially, decrease their breeding success and, for a reintroduction to be successful, individuals released for conservation purposes must not only survive but also must breed (Gilligan & Frankham, 2003; Mathews et al., 2005).

The colour distance analysis using a model of snake and a lizard visual systems also showed high differences on the skin coloration of frogs from Mistinjo breeding centre and wild populations, suggesting that predators could be able to perceive the different

colourations. Potential predators for the golden mantella frogs would be reptile species such as *Zonosaurus madagascariensis* and *Tamnosophis lateralis* (Jovanovic et al., 2009). Aposematism is a vital anti-predator strategy, it communicates to a potential predator unpalatability via visible traits (Maan & Cummings, 2012; Dreher, Cummings & Pruhl, 2015). During this study, it was not tested if potential predators would show a preference ofr captive frogs if given the opportunity. Further opportunities for understanding the function of their coloration in an antipredator context could be investigated in plastic model experiments that determine their natural predators and the protective value of their coloration.

The *M. aurantiaca* is a critically endangered frog with reintroduction as part of its Species' Action Plan to help mitigate the environmental impacts on the species' natural distribution (Edmonds et al., 2015). It is important to take the present results into account when considering releasing *M. aurantiaca* back to the wild, the aposematic coloration exhibited by these frogs has an important role in the species' behaviour and ecology. This is an important factor to be considered, however research still needed to understand if wild frogs and potential predators would still recognize captive frogs as the same species, regarding the changes on colouration. Captive animals must be in good physical condition to have a proper chance to survival and reproduce in the wild if they are to be released.

Chapter 6 - Comparing the bacterial communities of wild and captive golden mantella frogs: Implications for amphibian conservation

6.1 Abstract

Bacterial communities are frequently found in symbiotic associations with all animal species. The characteristically moist amphibian skin provides a good environment for the growth of bacteria; these bacteria can act as a first line defence mechanism against infections. Amphibians in the wild have relatively high exposure to bacteria through environmental transmission and through interactions with different individuals. Whilst in captivity animals interact with fewer individuals, as well as experiencing a less complex environment through which to obtain their bacterial community. During this research, we compared the skin microbiota of captive and wild *Mantella aurantiaca*, to investigate whether the captive environment was affecting bacteria associated with the skin. Skin microbiota was collected using swabs and a **c**ulture-independent methodology was used for the characterization of the microbial community through 16S amplicon sequencing methodology. Analyses showed that, even though captive individuals had significantly lower diversity of bacterial species and less abundant microbiota when compared to wild populations, some genus known to be involved on host health and disease protection were found on captive and wild populations. If the simpler bacteria community on captive frogs had their functionality affected, this could potentially impact their survivorship subsequent to a reintroduction .

Keywords: microbiota, amphibians, conservation, mantella

6.2 Introduction

The global amphibian crisis has resulted in an increased use of captive breeding as a conservation tool for amphibians (Griffiths & Pavajeau, 2008). Maintaining captive populations is important in terms of species conservation for potential reintroduction into the wild (Harding, Griffiths & Pavajeau, 2016). However, there is evidence that the captive environment can have negative impacts on different aspects of amphibians' ecology and behaviour, such as affecting their vocalizations (Passos, Garcia & Young, 2017a), anti-predator responses (Passos, Garcia & Young, 2017b) and skin microbiota (Loudon et al., 2014; Kueneman et al., 2016), which could potentially affect the survival chances of released animals.

The microbiota of amphibian skin is the first defence mechanism this group has against infections (Harris et al., 2009; Antwis et al., 2014a; Becker et al., 2014; Sabino-Pinto et al., 2016). Therefore, the proper functioning of this symbiotic interaction between bacteria and amphibians is vital for captive individuals, which are due to be released back into the wild (Antwis et al., 2014a). To understand whether captive bred frogs are fit for reintroduction the skin microbiota of wild and captive frogs of the same species needs to be compared, few studies have been done. Antwis et al. (2014a) observed changes in the richness and abundance microbiota of captive *Agalychnis callidryas* when compared to their wild counterparts and, a similar result was also found in six species of Japanese amphibians (Sabino-Pinto et al. 2016) and for the Panamanian golden frog, *Atelopus zeteki* (Becker et al., 2014). Different studies have demonstrated the effect of captivity on the loss of skin-associated bacteria on frogs and increased chances of infections (Loudon et al., 2014; Kueneman et al., 2016).

Bacterial communities are commonly found in symbiotic associations with most animal species (Küng et al., 2014; Walke et al., 2014). Frequently, the bacterial community provides some sort of advantage to the host such as protection against pathogens (Harris et al., 2009), and in return, receives nutrients and a suitable microhabitat in which to live and reproduce (Sabino-Pinto et al., 2016). The characteristically-moist amphibian skin surface provides a fertile environment for the growth of bacteria (Walke et al., 2014), some of which may be present throughout the life of the organism, and some of which are continuously exchanged between the skin and the animal's environment (Culp, Falkinham & Belden, 2007). These symbiotic bacterial communities contribute to the innate immunity of the host amphibian via competitive interactions between species and the production of antimicrobial metabolites, which are able to control the growth of some potential pathogens (Clarke, 1997; Antwis et al., 2015). Thus, they play an important role in protecting amphibians from infectious diseases, such as chytridiomycosis caused by the virulent and pathogenic *Batrachochytrium dendrobatidis* fungus (Cramp et al., 2014; Antwis et al., 2014). This disease has already been found in different areas in Madagascar, the natural habitat of our study species (Bletz et al., 2015).

Amphibians in the wild have relatively high exposure to bacteria through environmental transmission and through interactions with both conspecifics and other species (Walke et al., 2011). Amphibians in captivity interact with fewer individuals, as well as living in a less complex environment in which to obtain a rich and diverse bacterial community (Antwis et al., 2014a). Husbandry guidelines for keeping amphibians include removing waste, cleaning substrate and using a bleach dilution on enclosures to avoid the risk of diseases, but this could lead to a more sterile environment (Poole & Grow, 2012). Consequently, captive amphibians are likely to be exposed to a lower diversity of bacteria, and thus support a much simpler skin-associated bacterial community in comparison to their

wild counterparts. This could potentially make them less resistant to diseases when being reintroduced to the wild environment (Antwis et al., 2014a; Kueneman et al., 2016).

During this research, we analysed how the unique set of conditions created by captive husbandry conditions may affect golden mantella frogs' (*Mantella aurantiaca)* skin microbial colonization (Griffiths & Pavajeau, 2008; Harding et al.,2015; Passos et al., 2017a). From this, we predicted that captive bred frogs will have less abundant and rich skin microbiota, understanding this pattern is important because previous studies have shown reduced survivorship potential resulting from simplified skin microbial communities (Harris et al.,2009; Kueneman et al., 2016).

6.3 Methods

6.3.1 Study subjects

The study subject was the golden mantella frog, for more details please see section 1.8.

6.3.2 Study sites

The data used for this study were obtained from captive (Chester Zoo, UK) and wild populations (two spatially independent wild populations of frogs). The captive colony has been in captive for more than 7 generations. Frogs are kept off show in a biosecurity container specifically for conservation-related research. Frogs are kept in a group (same tank) of 16 individuals (10 males and 6 females), in a naturalistic tank with different live species of plants, moss for substrate, water, hiding places under rocks, UV light and heaters to mimic the natural conditions found in Madagascar. Tanks are cleaned monthly using diluted total spectrum disinfectant (F10®, Loughborough, UK). Wild frogs were sampled from Mangabe rainforest, a site of international biodiversity importance, home to most of the world's breeding ponds for the golden mantella frog. The second wild population was from Ambatovy mining site, located within a species-rich region of Madagascar at the southern

end of the remaining Eastern Forest Corridor in the Moramanga region. As part of the Environmental Management Plan, there is a Conservation Zone of native forest maintained by the mining company. For more details, please see section 1.9.

6.3.3 Skin Bacteria Sampling and DNA Extraction

To analyse the microbiota composition on the skin of golden mantella frogs a standard protocol described by Antwis et al. (2014a) was followed. Sterile gloves were worn throughout handling and changed for each frog to minimise the risk of cross-contamination. Prior to specimen sampling, frogs had their dorsal surface rinsed using sterile bottled water to remove any transient bacteria from their skin and ensure that the skin sampled included primarily skin-associated microbes. Frogs were then swabbed for 20 seconds all over the entire body surface and limbs to collect cutaneous bacterial communities using sterile cotton-tipped collection swabs (Figure 25). Swabs were kept in Eppendorf tubes with 200 µl of ATL buffer, another 400 µl of ATL buffer were added and samples were incubated for two weeks prior to DNA extraction. Care was taken to ensure frogs were not harmed during this process, individuals were kept in a plastic container after sampling to be monitored post-swabbing for signs of distress or injury in response to the swabbing (no adverse effects were observed) and to avoid re-sampling animals. After swabbing animals had their snout-vent length measured and body mass measured to allow for assessment of body condition a standard measure of amphibian health (Peig & Green, 2009).

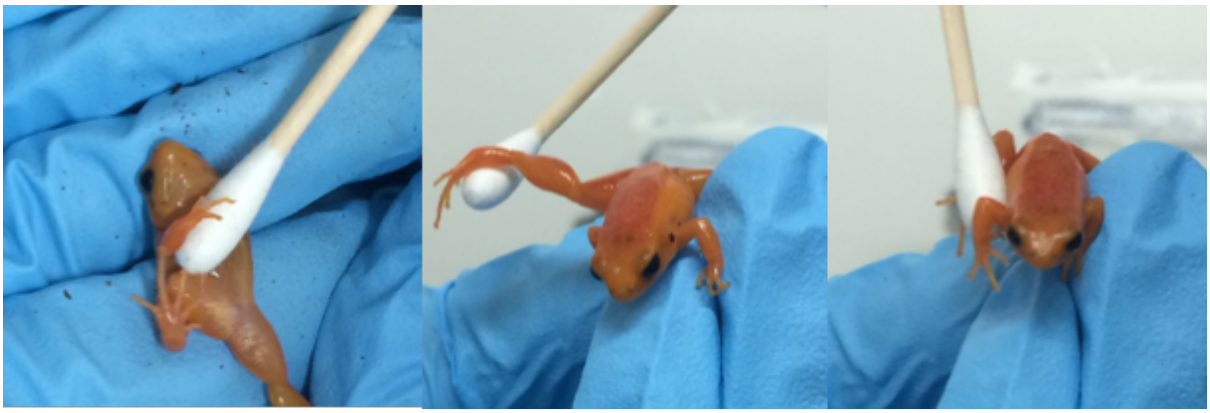

Figure 25. Golden Mantellas frogs having their body surface swab to collect skin-associated microbes (Photos by: Gerardo Garcia).

6.3.4 16S Metagenomic Sequencing

During this study, a culture-independent methodology was used for the characterization of the skin associated microbial community. A total of eight individuals from each population (4 males and 4 females) were used for the molecular analysis. DNA was extracted from the swabs using QIAGEN DNeasy Tissue and Blood kit. The standard QIAGEN protocol for swab samples was followed with modifications for samples with low quantities of DNA. Adjustments included 24 hours incubation in 56 °C after the addition of ATL buffer and Proteinase K. Addition of 4 µl of RNAse before adding AL Buffer and allowing AE buffer to sit on the filter for 20 min before the final elution (Lauber et al., 2010). To confirm the presence of DNA on the extraction's product, an agarose gel was used. A NanoDrop spectrophotometer was used to determine the purity and DNA concentration of this pool.

Library preparation was done following the MiSeq 16S library preparation two step PCR Illumina protocol. Bacterial species isolated from captive and wild populations of *M. aurantiaca* were identified using the 16s Illumina amplicon protocol with primers 515F-806R (FWD:GTGCCAGCMGCCGCGGTAA; REV:GGACTACHVGGGTWTCTAAT) targeting the V4 region. 16S DNA was amplified using a two stage PCR with a HotStart PCR kit

(Kappa Biosystem) following the manufacturer's instructions. First stage PCR with the following program: 95°C for 3 min followed by 25 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s, with a final extension step of 5 minutes at 72°C. PCR products were checked for the correct length using a Tape Station Screen Tape High sensitivity (Agilent) and then cleaned up using AMPure XP beads was used to remove primer dimers. A second stage PCR with the following program: 95°C for 3 min followed by 8 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s, with a final extension step of 5 minutes at 72°C. PCR products were again checked for the correct length using a Tape Station Screen Tape High sensitivity (Agilent) and then cleaned up using AMPure XP beads (Agentcourt) to remove any unwanted DNA. Qubit Fluorometric Quantitation (Thermo Fisher Scientific) was used to determine the purity and DNA concentration of each sample. Samples were pooled together and a qPCR using NEBNext Library Quantification Kit (Illumina) was performed to quantify library DNA concentration. The library was loaded in the MiSeq Illumina V2 reagent cartridge with 30% PhiX (Illumina) as control. A consensus sequence was obtained by combining the forward and reverse sequences and processed with the R package dada2 pipeline using the default parameters (Callahan et al., 2016). Consensus sequences were then blasted against the Ribosomal Database Project (RDP; http://rdp.cme.msu.edu/) to identify each morphotype to genus level.

6.3.5 Statistical Analysis

We used R packages Phyloseq (McMurdie & Holmes, 2013) and DESeq2 (Love et al., 2014) to identify differences in the abundance of bacterial taxa between treatment groups. Libraries filtered out all OTUs with <20 reads and rarefied to 9000 reads per samples following protocol in Longo & Zamudio (2017). Species alpha diversity was obtained using

the Shannon-Wiener metric and compared between populations, and wild versus captive samples.

For the relative abundance for beta diversity analysis, the overall bacterial community composition was analysed for differences based on origin (wild versus captive) and population using the Adonis function of the vegan package (Dixon, 2003) in RStudio (2015). Adonis is a permutational multivariate analysis that uses a Bray-Curtis distance matrix based on the abundance of each morphotype to analyse the variation in the overall bacterial community structure. The effect of origin and population on species richness and total abundance (mean number of sequences per sample) were analysed using a one-way ANOVA followed by a *post-hoc* Tukey test, the effect of gender and body condition was analysed using a T-test, all tests were conducted in Rstudio (data for bacterial abundance were log transformed to achieve a normal distribution).

Differential abundant analyses were conducted using the R package DESeq2 (Love et al., 2014), which allows quantitative estimates of differences in bacterial abundance in different populations without the bias of rarefying libraries (McMurdie & Holmes, 2014). OTU abundance between the three populations and between wild and captive populations were quantified using Wald tests, a Bonferoni test was applied to correct p-values due to multiple testing.

6. 4 Results

Analyses from the sequencing data showed 563 (Appendix) different morphotypes belonging to 153 genera, 98 families, 66 orders, 39 classes and 20 phyla (Table 13). The average number of sequences per sample was 14779±365 for Ambatovy samples, 17155± 419 for Mangabe samples and 9435±215 for samples from Chester Zoo. Two hundred and seventy-two morphotypes were isolated from Ambatovy (wild) samples, 206 morphotypes

were isolated from the Mangabe (wild) population and only 100 morphotypes from frogs kept at Chester Zoo. Some morphotypes, across all populations, could not be identified due to poor a sequence. Morphotypes with a sequence similarity of 99% or greater were considered the same species.

Table 13. Number of phyla, classes, orders, families and genera identified per golden mantella frog population.

Population	Origin	Phyla	Class	Order	Family	Genus
Ambatovy	Wild	11	21	38	65	87
Mangabe	Wild	20	39	60	84	114
Chester Zoo	Captive	9	15	23	34	40

Only ten bacterial genera (*Acinetobacter, Pseudomonas, Bradyrhizobium, Dokdonella, Enterobacter, Providencia, Rubrobacter, Salmonella, Serratia and Spirosomo*) from six different families were isolated from both wild and captive populations (Figure 26). One family of bacteria, Enterobacteriaceae, comprised a greater percentage of reads from both wild and captive *M. aurantiaca,* being the most abundant family (85% Mangabe, 76% Ambatovy and 60% Chester Zoo) (Figure 27) when compared to other families.

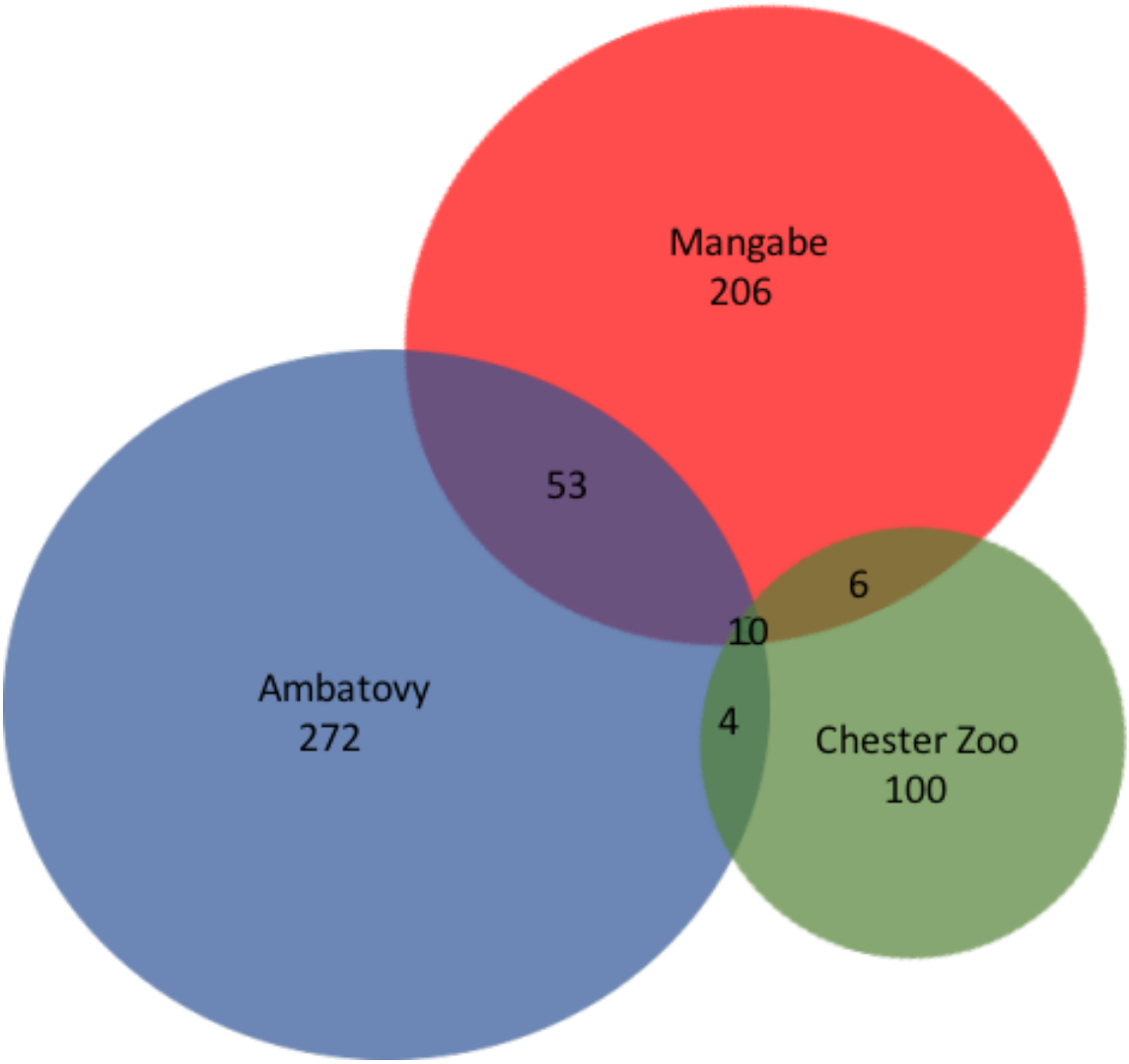

Figure 26. OTUs unique to and shared between the golden mantella frogs from the three sampled populations, Mangabe, Ambatovy and Chester Zoo.

Wild frogs had a significantly higher skin bacterial alpha diversity than those reared at Chester Zoo (wild Shannon-Wiener index= 56.55, captive Shannon-Wiener index= 11.83, $p<0.05$). When alpha diversity was compared between the three groups, Mangabe (Shannon-

Wiener index = 38.61) had the greatest diversity between all sampled populations (Ambatovy Shannon-Wiener index = 23.07, Chester Shannon-Wiener index = 11.83, $p<0.001$)

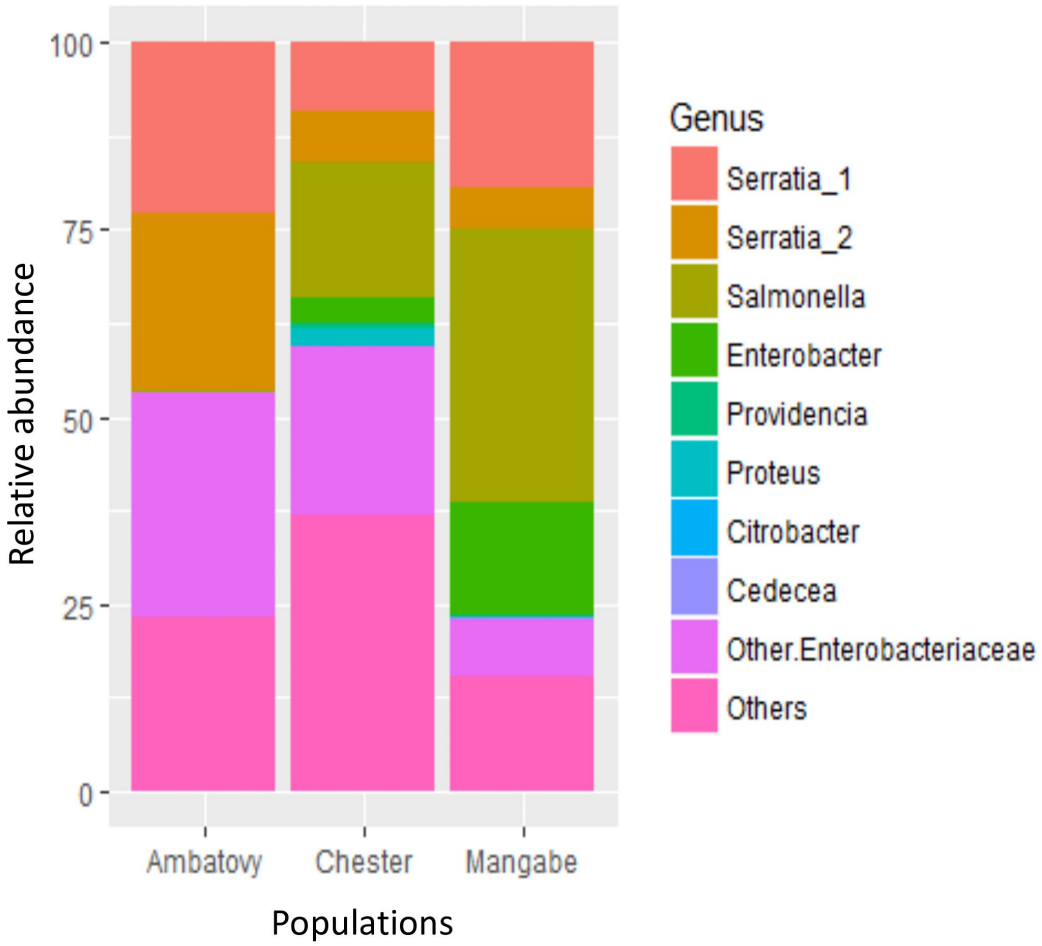

Figure 27. The relative abundance of sequences assigned to major bacterial family (Entebobacteriacea) observed in each of the golden mantella frog populations.

The Adonis model showed that origin (wild versus captive) ($F_{1,23}$=4.02, R^2=7.20, p<0.001) and population (i.e. Ambatovy, Chester Zoo and Mangabe) ($F_{2,22}$=2.84, R^2=7.71, p<0.001) had a significant effect on the overall bacterial community composition associated with frogs (Figure 28). Neither the sex of frogs nor their body condition had a significant effect on bacterial abundance or species richness when comparing wild and captive animals or in each population separately (p>0.05 in all cases).

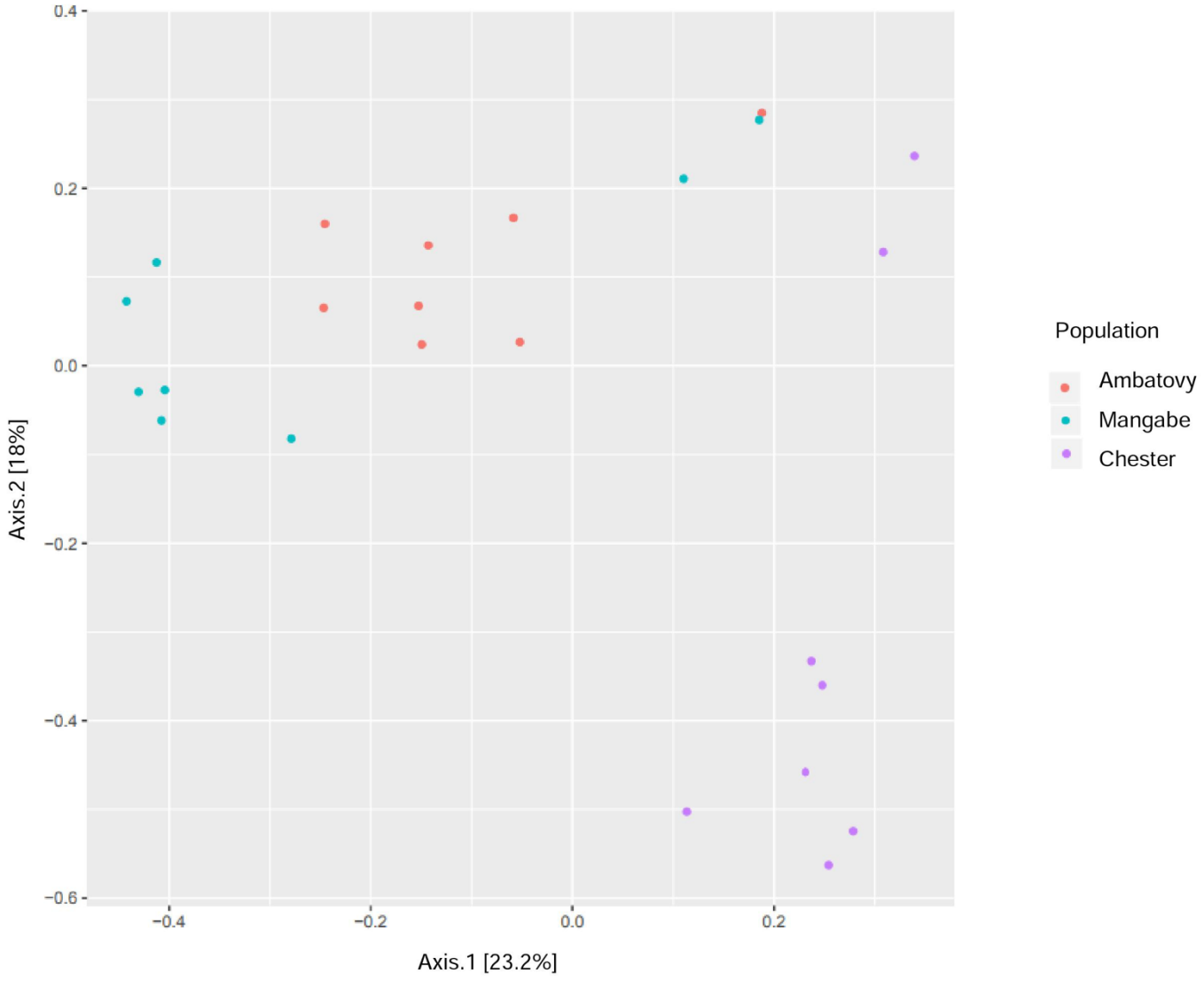

Figure 28. Plots from two-dimensional MDS analyses representing the population-related differences in the composition of the skin bacterial communities of golden mantellas frogs.

Differential abundant analyses using DESeq2 on the unrarefied data set identified 209 OTUs, which were more abundant in Mangabe, 90 that were more abundant in Ambatovy and only 5 that were more abundant in Chester (Figure 29).

Figure 29. Heatmap showing the 32 most abundant OTUs on the three studies population, Chester Zoo, Ambatovy and Mangabe.

6.5 Discussion

During this study, we observed that golden mantella frogs kept in captivity presented significantly simpler skin microbiota in comparison to wild conspecifics. This result was expected considering previous studies that also found similar results with captive colonies having a less rich and abundant skin-associated microbiota (Antwis et al., 2014a; Becker et al., 2014; Kueneman et al., 2016; Loudon et al., 2014; Sabino-Pinto et al., 2016). Given the important role symbiotic microbiome communities have for the innate immunity of the host amphibian (Woodhams et al., 2014), the findings of this study are important for conservation.

Bacterial communities of the skin of captive and wild golden mantella frogs were dominated by Gamma-Proteobacteria, and Actinobacteria, which is in agreement with findings from amphibian studies in North America (Kueneman et al., 2014), Central America (Rebollar et al., 2016), Europe (Vences et al., 2015) and Japan (Sabino-Pinto et al., 2016). The composition of microbiomes associated with amphibians skin is determined by a diversity of factors, and disentangling these is challenging (Sabino-Pinto et al., 2016). The less diverse bacterial community in captive may lead to a higher susceptibility of the frogs to diseases (Becker & Harris, 2010), if important symbiotic bacteria were missing. Therefore, this needs to be considered for *ex situ* management of threatened amphibians, especially in projects that have as their objective the reintroduction of individuals to the wild (Passos, Garcia & Young, 2017b).

Studies suggest that the structure of the microbial communities can have direct impacts on their function, and ultimately on host phenotype (Becker et al., 2014; Sabino-Pinto et al., 2016). Communities that are richer in species are likely to have an increased ability to produce antifungal metabolites and, as a result, protect their hosts against infections

(Loudon et al., 2016). Several studies have already provided evidence consistent with a correlation between overall microbiome diversity and susceptibility to infectious disease and costs associated with host responses to pathogen exposure (Kueneman et al 2016, Harrison et al., 2017). The less rich community with a different composition observed on the captive colony when compare to the wild populations could be less efficient in protecting their host against pathogens.

Strains of the genus *Janthinobacterium* and *Pedobacter* were found in samples from golden mantellas sampled in Mangabe (wild). Despite being found in low abundance it is important to emphasize that some bacteria species from these genera have already been identified as chytrid inhibitory (Harris et al., 2009). If these bacteria occur naturally on the golden mantella frogs' then wild individuals, potentially, have a natural resistance to this fungus. Most available studies focus on the more abundant members of the bacterial communities, but future work on rare morphotypes is necessary because these could have important roles for host health (Kueneman et al., 2016).

Furthermore, members of the genera *Pseudomona*s and *Acinetobacter* were found on all three studied populations, these genus, which are commonly found in the skin of other tropical and temperate amphibian species, are known to play a role in host health and disease protection (Loudon et al. 2014b; Kueneman et al. 2014; Walke et al. 2014). This shows that even though captive frogs have a simpler bacterial composition on their skin, is it possible that this microbiome still retains its functionality against pathogens.

Microbiota reservoirs (e.g., water, soil, and plants) appear to be sources of skin microbiome for frogs, and host internal drivers might help sculpt the composition of these communities (Nyholm et al., 2000; Michaels et al. 2014; Loudon et al. 2014b; Walke et al. 2014; Becker et al., 2015; Loudon et al. 2016; Sabino-Pinto et al. 2016; Kueneman et al.

2016). Captive environments are less complex than wild environments and, are routinely cleaned by keepers, with water drained and substrate changed (Edmonds et al., 2015). This could prevent bacteria colonies from developing and, consequently, associating with the frogs' skin (Cramp et al., 2014). Different studies also suggest that amphibians could have obtained their skin bacteria through vertical (parent to offspring) or horizontal (between individuals) transmission (Walke et al. 2014). The more interactions, more bacteria would be transferred increasing abundance and richness (Walke et al., 2011). In captive frog colonies, individuals would be always interacting with the same individuals, decreasing the chance of exchanging different bacteria (Antwis et al., 2014a); this could help explain why frogs from Chester Zoo had a less rich microbiota when compared to wild populations. However, mechanisms of bacterial transmission in amphibians have been barely explored (Walke et al. 2014; Rebollar et al. 2016).

The main concern about the species poor bacterial community on the skin of captive golden mantella frogs was related to the plans for reintroduction of captive bred individuals to the wild. The lack of some bacteria species could prevent individuals from being able to resist some natural pathogens in the wild (Sabino-Pinto et al., 2016). Recent studies have already detected the presence of the amphibian chytrid fungus, in wild populations of amphibians in Madagascar, including regions near the golden mantella occurrence (Bletz et al., 2015). Although there is still a lack of clinical signs of chytridiomycosis in Madagascar, the recent arrival of a virulent chytrid lineage to the country is expected to have some negative effects on the frog community (Bletz et al., 2015). Ongoing studies are trying to discover how to improve the host bacteria assemblage using probiotics (Küng et al., 2014; Antwis et al., 2015; Vence & Raxworthy, 2004). More research is required to investigate how bacterial communities change over time (generations) when host organisms are brought into captivity, and how this may affect their susceptibility to disease (Antwis et al., 2014a).

There are still many factors to be considered to understand the dynamics of amphibian skin associated bacterial communities, their composition and variation, and the development of methods to maintain and manipulate it, could be fundamental for conservation management of captive and wild amphibian populations (Sabino-Pinto et al., 2016).

Chapter 7 - General Discussion

During the attempt to the proposed questions in this thesis, more questions were generated regarding the possible consequences of captivity. In the first chapter, it was observed that captivity has affected the vocalizations of golden mantella frogs, this effect seems to increase with the duration of animals stay in captivity. Phonotaxis experiment demonstrated that captive frogs took a longer and less accurate path to find the sound source. However, they had a faster and more accurate response to their own calls, whilst wild frogs would recognize and respond to captive and wild call in a similar way. The difference in the captive frogs' responses could be associated with lack of species recognition or the "dear enemy effect" where males do not respond to "familiar neighbours" with aggression.

A second experiment, using the same pre-recorded calls, with captive frogs lead to a different result. The playback experiment performed with Chester Zoo's captive colony had animals showing an increase in activity, especially breeding associated behaviours, when wild calls were being played, such response was not observed when captive calls were being played. Captive calls could be lacking essential traits to stimulate breeding behaviour. Comparing the results of both playback and phonotaxis experiments it is possible to assume that captive animals do recognize wild calls, despite being captive for over 7 generations. Although wild frogs also recognized captive calls during phonotaxis experiments, it is still necessary to evaluate if the changes in different call parameters would affect the breeding success of captive frogs, if released back to the wild.

Anti-predator responses are especially important to maintain in captive animals if they are being bred for reintroduction. In this chapter, a tonic immobility test was done with two captive colonies were compared to results obtained from wild frogs. Results showed that Chester Zoo frogs had responses with no statistical differences when compared to wild ones.

Frogs kept at Mitisinjo Breeding Centre had shorter responses when compared to wild and Chester Zoo animals, with many individuals not presenting TI responses, this result was, however, cofounded with a lower body condition.

The fourth chapter of this thesis was an analysis of the skin colouration of captive and wild frogs in an attempt to determine if there was a significant difference and, to quantify the observed changes. The spectrometry analysis did find a difference between captive and wild frogs, however, a more in depth investigation using a colour distance analysis showed that the difference found between Chester Zoo and wild animals was low to non-detectable, while between Mitisinjo and wild animals was extremely high.

The last research chapter was an analysis of the microbial community found on golden mantella frogs' skin. Symbiotic bacteria found on amphibian skin have an important role as a defence against pathogens. What we observed was a less rich and abundant microbial community in captive frogs, although some important genus were present in all studied populations. The extent that these changes would have on the survivorship of these frogs if they were to be released in to the wild is hard to predict, but could be negative.

Many of the parameters studied in this thesis were related to intra- and inter-species communication. The ability to communicate to conspecifics and other species is essential for survival and for reproductive success. Other factors, such as TI and skin microbial community, are related to surviving potential threats such as predators and pathogens, which are common in their natural environment.

It is important to state that Chester Zoo's frogs are not kept for conservation purposes including reintroduction. The population is kept for research purposes and their husbandry is a reflection of this. Animals that are part of a breeding programme would have a different genetic management to maintain their genetic diversity. However, Chester Zoo's colony was

use as a model kept under stringent hygiene conditions. Mitsinjo's golden mantella frog colony is being bred for reintroduction, making the results presented here more meaningful, because some important effects of captivity that could affect frog survivorship after release were observed.

Strategic use of captive breeding can be a potent tool for species conservation that complements field conservation (Harding et al., 2015). Potential *ex situ* goals, objectives and actions should therefore be evaluated alongside field activities in the process of conservation planning, to ensure that they are used appropriately (IUCN/SSC, 2014). When planning, it is important to consider the roles an *ex situ* programme can play, the characteristics and dimensions it should take, and what factors will impede or likely contribute to conservation success (IUCN/SSC, 2014). Although zoos are conservation institutions, however individuals housed at breeding facilities for reintroduction must have the necessary skills to be returned to the wild, survive and breed.

The IUCN reintroduction guidelines states that captive individuals should be from populations with appropriate demographic, genetic, welfare and health management (IUCN/SSC, 2013). Released animals should exhibit behaviours essential for survival and reproduction, and for compatibility with any conspecifics in the release area. It may sometimes be desirable to move groups of animals with their social relationships intact (IUCN/SSC, 2013). Reintroductions are costly and time consuming; therefore, to make the best use of resources available it is important to pre-release screen individuals, which are destined for reintroduction (Canessa et al., 2016).

Reintroduced animals must not only survive, but also reproduce (Kraaijeveld-Smit et al., 2006), which involves finding a suitable mate, and be able to communicate with such conspecifics (Christie et al., 2010). Communication is an important skill, and different

communication signals are used by golden mantella frogs, such as skin colouration (Ogilvy et al., 2012) and vocal calling pattern (Caldart et al., 2016). Both were affected by the captive conditions as demonstrated in this thesis, if released animals cannot communicate with their wild conspecifics, then the chances of reproduction with wild individuals can be reduced (Christie et al., 2010). Reproductive success is linked with males outcompeting other males during sexual selection. Skin colouration and vocalizations are used during courtship behaviour, releasing animals will these characteristics affected by captive conditions could reduce their breeding success. The communication traits used by golden mantella frogs are not exclusive for this species, hundreds of species use skin coloration patterns for intra and interspecific communication and, most frog species have vocalizations as part of their breeding behaviour (Sun & Narins, 2005), thus effects observed during this study could be extrapolated to other species, which are being kept in captivity for conservation purposes.

One of the captivity consequences observed during this study was the change in the vocal call structure and temporal patterning of calls by captive golden mantella frogs. We observed that frogs kept in captivity for longer time periods (more generations) had greater changes in their calls. Vocalisations are a result of the acoustic environment in which the species is found (Caldart et al., 2016; Sun & Narins, 2005) with animals shaping their calls to better adjust to the background noise of their environment (Brumm & Slabbekoorn, 2005; Scofield et al., 2011). One remaining question is: If a call that is shaped for anthropogenic background noise will also be effective in a wild setting?

Studies have shown the ability of different taxa to modify their calls as a consequence of changes in the background noise (Brumm & Slabbekoorn, 2005; Caldart et al., 2016). Such short-term vocal adaptations have been examined across different taxa, such as insects, anurans, birds, and mammals (Brumm & Slabbekoorn, 2005). Now is important to comprehend how these modifications happens, if they are permanent and how to mitigate

them.

An increasing number of studies show that captive breeding can result in rapid selection or plastic responses in phenotypic or life-history traits that can reduce an individual's fitness upon release to the wild and compromise the chances of successful reintroduction (Slade et al., 2014). During this study, we were able to demonstrate that the vocalisations of first generation of captive golden mantella frogs were already significantly modified in captivity. The inability to communicate with wild conspecifics could negatively impact conservation efforts involved in a reintroduction programme (Gilligan & Frankham, 2003; Mathew et al., 2005).

Another possible communication path is through the use of visual signs such as skin colouration (Siddiqi et al., 2004). Skin colouration is an important anti-predator strategy, through aposematic signals (indicating unpalatability) from prey to a predator (Dreher et al., 2015). Skin colouration is also important for intra-specific communication during the breeding season (Siddiqi et al., 2004). In this study we observed that captive golden mantella frogs presented a duller colouration when compared to wild individuals, especially golden mantella frogs from Mitsinjo captive breeding centre in Madagascar. Many amphibian species use carotenoids for skin pigmentation, and because carotenoids are only obtainable through the diet, colour degradation could result from limited carotenoid availability (Ogilvy et al., 2012). However, colour change due captive conditions could be reversed with changes in husbandry such as increasing UV light levels and carotenoid diet supplementation (Ogilvy et al., 2012). Studies have shown that frogs fed with a carotenoid enriched diet had a significantly redder skin than individuals without (Ogilvy et al., 2012).

One of the many challenges that frogs will have to face after reintroduction is predators (Kraaijeveld-Smit et al., 2006). Captive animals are naïve to predators due to a lack

of experience with them, resulting in failure to recognize a species as a predator and the adoption of inappropriate antipredator behaviour (Moseby et al., 2012). Captive environments are highly predictable and lack threatening situations that could lead to important defensive responses weakening or disappearing across generations of captive breeding (Kraaijeveld-Smit et al., 2006; Teixeira et al., 2007). However, animals, including amphibians, can be behaviourally conditioned before release to avoid predators or to develop antipredator skills (Maloney & McLean, 1995; IUCN/SSC, 2013). Where possible, practitioners should design experiments to determine the efficacy of conditioning techniques and/or to determine correlates between pre-release behaviour and post-release survival (IUCN/SSC, 2013).

Antipredator training, therefore, may be a valuable addition to reintroduction programs (Maloney & McLean, 1995) and could be a useful strategy to increase translocation success (McLean 1996; McLean et al., 2000; Azevedo & Young 2006; Mesquita & Young 2007; Teixeira et al., 2007). Tonic immobility (TI) experiments demonstrated that anti-predator responses can be maintained in captive populations and assessed with simple experiments. The experiments also showed that the lack of TI responses could also be related to health (body) conditions, making this test even more relevant for reintroduction programmes.

Besides struggling for survival against predators, captive individuals also face a different threat: parasites and infections. Disease risks are an important factor when planning a reintroduction (IUCN/SSC, 2013). Animals in the wild are exposed to a wide range of microorganisms and parasites that captive animals have not yet experienced (Walke et al., 2011). Having the appropriate immune system to fight these pathogens is essential for survival. The microbiome community associated with the skin of amphibians is an important part of the amphibian immune system, having a great impact on host susceptibility to a range

of infectious diseases (Jani & Briggs, 2014). Amphibians in the wild gain skin bacteria through environmental transmission and through interactions with conspecifics and other species. Captive amphibians have limited intra-specific interactions, as well as being kept in a less diverse environment through which to gain bacteria. The impact of these differences, is that captive frogs having a less rich and abundant skin microbiota, as was observed in this study making them less resistant to disease (Jani & Briggs, 2014).

After carefully analysing the results obtained during this study it is possible to show that individuals can be affected by captive conditions and that pre-release screening is necessary to understand these consequences and, thereby, increase the success rate of reintroductions. There is an increasing need to ensure the integration of *in situ* and *ex situ* conservation planning to guarantee that, whenever appropriate, *ex situ* conservation is used to support *in situ* conservation to the best possible effect (IUCN/SSC, 2014). Knowledge of the species' natural habitat (humidity, light levels, etc.), diet and behaviour collected during field work could help elucidate the gaps in husbandry and how to better maintain animals in captivity to avoid deleterious effects related to captive breeding.

7.1 Research limitation

Data collected during this project supports the idea that wild animals can be negatively affected by captive conditions in terms of their conservation. However, this study was focused on the golden mantella frogs and, not all the results found can be extrapolated to all species of frogs. The results presented here, can and should be use as a guideline to new research focus on the effects of captivity in amphibians' characteristics, thus attention should be given to the ecological needs of the species in question when in a captive breeding programme.

Our findings also show that husbandry can have a great impact on how much the conservation potential of animals are affected by captivity. Captivity does affect animals, but results cannot be generalized as the husbandry regimes in each captive breeding centre tend to vary. One means to improve this study would have been to have included more captive populations, but this was not possible due to logistical constraints. Despite this, the results presented here could be used to contribute to harmonising husbandry procedures across institutions.

Due to time and financial constraints, it was not possible to test different alternatives to mitigate problems observed during the research, such as lower body condition, affected calls or different skin colouration, or how much all the observed changes on captive golden mantella frogs would compromise their survival during a reintroduction. During this study, we did not attempt to make any changes on the husbandry regime that captive frogs were exposed to, instead we evaluated frogs in the conditions under which they are currently being kept. It is important to understand if these effects are actually permanent and, if not, how long animals would need during a soft-release (reintroduction) to adjust back to the wildtype behaviour, physical condition, etc.

Finally one of our sampled wild populations of golden mantella frogs was from a site impacted by mining activity and we assumed that these individuals represented healthy wild models. This assumption is supported by our comparisons with wild individuals sampled from the conservation zone in Madagascar who were not significantly different in any of the comparisons that we made. The conservation zone was very well conserved.

There are many others important traits that should also be addressed when evaluating the fitness of captive populations such as genetic diversity, prey recognition, adequate predatory response and habitat selection, which due to time constrains were not evaluated

during this study. As with many scientific studies I have, probably, alerted science to more questions than I have answered, however, I have tried to address questions, which I consider important in terms of species conservation. I have focussed on avoiding predation (tonic immobility response and correct colour), attracting a mate (vocalisations and correct colour), general fitness (body condition) and ability to fight off infections (i.e. skin microbiota) because I believe these to be some of the most important characteristics of animals destined for reintroduction.

Future research

The results obtained during this project shows that frogs' behaviour and ecology were altered by husbandry and captive conditions and, that pre-release screening could increase the success rate of reintroductions. It is also necessary to find methods to mitigate these effects of captivity prior to any release attempt. Reintroductions are inherently risky because they require the movement of individuals from a relatively secure environment (such as a zoo or stable wild population) to an environment that was previously unable to sustain a natural population (IUCN–SSC, 2013).

The next stage for this research would be to test the effectiveness of soft-release as a mitigation measure. This method may increase the likelihood of reintroduction success because individuals are forced to acclimatize and become familiar with the new release site prior to permanent release (Attum & Rabia, 2016). It would be possible to closely monitor captive animals (Milliano et al., 2016) and understand the aspects in which released individuals would struggle to adapt in their new environment and act on it before any deleterious consequences. With the right support (e.g. food supplementation, shelter and protection against predators) animals could have enough time to adjust to the wild conditions (Miller at al., 1999). If, in a soft release, the behavioural and morphological differences

observed between captive and wild individuals diminished (Miller et al., 1999), then it could be a time and cost effective measure. However, experimental testing of soft-release methods is rare, despite being necessary.

Using the golden mantella frogs as an example, with access to their natural diet (Brenes-Soto & Dierenfeld, 2014) and appropriate UV light conditions (Michaels et al., 2014), maybe it would be possible to eliminate a few of the problems identified during this research. Skin colouration and body condition could, potentially, be reversed back to the same as their wild conspecifics (Ogilvy et al., 2012). The positive effect of soft-release on body mass has been demonstrated before with hard-release individuals losing mass (Bright & Morris, 1994). Animals that already have a low body mass would not endure a lower mass. Amphibian skin colouration, especially red tones are directly connected to carotenoids derived from their diet (Ogilvy et al., 2012, Dugas et al., 2013). Access to their natural diet could provide enough carotenoids to re-create their natural skin colouration (Brenes-Soto & Dierenfeld, 2014).

Vocal calls are shaped by the acoustic environment (Sun & Narins, 2005) and animals have the ability to change their vocalizations to better suit the background noise in they are exposed to (Brumm & Slabbekoorn, 2005). Returning animals back to the wild acoustic environment could, given the sufficient time, lead to calls being changed back to fit with the background noise of their natural habitat if vocalisations are sufficiently plastic.

Species recognition is very important during a reintroduction. It is necessary to test if captive frogs with a blunted colouration would not be recognized by wild individuals. Besides testing intra-specific recognition, it is also important to know if frogs with a dulled colouration would be seen as a prey by potential predators in their natural habitat.

A genetic evaluation of the captive and wild populations would be necessary to fully understand the genetic diversity involved on both groups. Relaxed natural selection (e.g., reduced competition and predation) via unintentional domestication can act to rapidly deplete genetic diversity in captive populations and may lead to an accumulation of deleterious mutations (Willoughby et al., 2015). The lack of genetic diversity associated with population size can result in population bottlenecks, which are associated with increased rates of inbreeding and even greater loss of genetic diversity, both of which can affect the long-term viability of reintroduced populations (Jamieson, 2011).

There is still much to be understood about how the frog skin's microbiota is obtained and the effects of changes on this bacteria community on the frog's health (Walke et al., 2011, Sabino-Pinto et al., 2016). We did not attempt to develop a probiotic to understand how to incorporate the right bacteria composition on frogs' skin, which would be necessary before releasing individuals back to the wild.

We have observed that many of the captive consequences were related to husbandry differences. Understanding the role that husbandry plays on how animals adjust to the captive environment is really important for the conservation of many amphibian species. For many species of amphibians a release date is far from being determined due to no short-term solution to the threats affecting them in their natural habitats (Harding et al., 2016). Another important factor to be studied is the evaluation of changes on husbandry regime and how this could improve the conditions of already impacted individuals, making them more similar to their wild conspecifics.

More research is necessary to fully understand the effects of captivity on other aspects of amphibian ecology such as dietary requirements, breeding behaviour, nest site selection, amongst others. It is also important to evaluate different abiotic variables (e.g. humidity,

temperature, etc) and how they influence different aspects of the frog's life cycle and behaviour. If animals are to be in captivity for many generations before having a chance to be reintroduced to the wild, it is even more important to understand best husbandry practices to keep captive frogs fit for the wild.

7.2 Conclusion

The use of captive breeding as a conservation strategy for amphibians has grown in importance in the last decade, especially due to the spread of the chytrid fungus disease. For many species, this is the only viable option in the short term and should be used to complement field conservation. After exploring different aspects of captive and wild golden mantella frogs it is possible to conclude that captivity breeding can be an important and viable option for the conservation of threatened amphibians. However, husbandry techniques can play a major role in attenuating or increasing these consequences of being in captivity. Nonetheless, special attention should be taken when choosing the husbandry regime to fulfil all the environmental and behavioural needs of each species. More research is still needed to fully understand the consequences of keeping species in captivity for many generations without contact with wild individuals.

Nevertheless, pre-releasing screening not only for health condition, but also for behavioural skills should be mandatory for reintroduction programmes. Releasing animals with their abilities to survive and reproduce compromised is unethical and would negate all the conservation effort involved in breeding and releasing animals back into their natural habitats. Such programs are costly and for this reason, it is imperative, to make sure that the resources are being applied in the best manner possible.

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Appendices

PLOS ONE

RESEARCH ARTICLE

Neglecting the call of the wild: Captive frogs like the sound of their own voice

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Abstract

Acoustic communication is highly influential in the expression of social behavior by anuran amphibians, transmitting information about the individual's physical condition and motivation. We studied the phonotactic (approach movements) responses of wild and captive male golden mantella frogs to conspecific wild and captive playback calls to determine the impact of captivity on social behaviour mediated by vocalisations. Calls were recorded from one wild and two captive populations. Phonotaxis experiments were then conducted by attracting *M. aurantiaca* males across a PVC grid on the forest floor or enclosure floor to a speaker. For each playback, the following parameters were recorded to define the accuracy of phonotaxis: (1) number of jumps; (2) jump angles; (3) jump distances; (4) path straightness. During this experiment we observed that wild frogs had a similar behavioural (phonotaxis) response to calls independent of their source while frogs from Chester Zoo had a significantly stronger response to calls of other conspecifics held separately at Chester Zoo. The lack of appropriate phonotaxis response by captive bred frogs to the calls of wild conspecifics could have serious negative conservation implications, if the captive bred individuals were released back to the wild.

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Introduction

Communication is the foundation upon which all social relationships between animals are built [1]. Acoustic communication is probably the most influential trait in the social behavior of anuran amphibians. Although the circumstances in which animals vocalize vary between species, virtually all male frogs incorporate some form of advertisement call into their vocal repertoire that is usually a necessary precursor to successful courtship and mating [2].

In anurans significant information about the individual's fitness is transmitted by acoustic signals [3,4]. Among male frogs, vocalisations allow the identification of the resource holding potential of an opponent [5,6], facilitate inter-male spacing [7,8] and permit the recognition of territorial neighbours [9]. Field experiments using playback calls have revealed that vocalisations also play an important role in sexual selection during male–male competition and female choice in many species [7,8,10,11].

Phonotaxis is defined as any kind of movement or orientation towards specific acoustic signals [12]. Positive response is taken as evidence of both perception and recognition of the

acoustic stimulus by the receiver [9]. It has been widely demonstrated that playback experiments are an adequate methodology to analyse phonotactic responses of frogs [11,12,13].

It is believed that the captive environment can significantly affect the vocalisations of animals to a point where their calls are no longer recognised by wild conspecifics [14]. This would of course have serious implications for reintroduction programmes [14,1]. Therefore, we studied the phonotactic responses of wild and captive male golden mantellas (*Mantella aurantiaca)* to conspecific wild and captive playback calls.

Methodology

Study subject

The golden mantella frog (*Mantella aurantiaca)* is a critically endangered species [15], found only in Madagascar with a distribution restricted to a fragment of forest that is under severe threat from mining, agriculture, timber extraction and over-collecting for the pet trade [16]. According to the Amphibian Ark, *ex-situ* assistance is vital for the long-term survival of the golden mantella frog [17].

Study sites

Golden mantellas calls were recorded from three different populations: wild calls from Mangabe, Madagascar and captive calls from Mitsinjo Captive Breeding Centre (located in Madagascar) and Chester Zoo (UK). The phonotaxis experiments were performed with wild frogs in Madagascar and from captive frogs kept at Chester Zoo.

Mangabe area (Madagascar). Mangabe also known as the "blue forest" is a site of international biodiversity importance, divided into two administrative districts, Moramanga in the north and Anosibe An'ala to the south. Data sampling for this study was done in the Moramanga region. Most breeding ponds for the golden mantellas frogs are found in this area according to recent studies concerning conservation priority sites for mantella frogs.

Chester Zoo (UK). The zoo currently maintains two visually and acoustically isolated *ex situ* groups of *M. aurantiaca*, one is on public display at the zoo's Tropical Realm exhibit from which calls were recorded and a second group is kept off show in a biosecurity container specifically designed for conservation-related research, where the playback experiment was conducted with these frogs. The biosecurity container is kept under temperature and humidity regimes to give the frogs a similar environment as they would experience in the wild. Enclosures are annually modified to keep animals under rainy and dry periods as per their natural environments.

Mitsinjo Association Captive Breeding Centre (Madagascar). This community-run conservation organisation operates around the village of Andasibe in east-central Madagascar and it holds the first Malagasy biosecure facility to protect endangered amphibians. Fifteen local species including a genetically viable population of the golden mantella frog taken from the wild (i.e., genetic founders) collected at the Ambatovy area, and their F1 offspring are currently being kept at Mitsinjo. Only calls from the F1 frogs were recorded and used (no playback experiments were done here).

Ethical approval

All the research reported in this study was approved by the Chester Zoo's Ethics Committee, UK and it conforms to all regulations and laws in all relevant countries in relation to care of experimental animal subjects. Furthermore we can confirm, from our post-experimental monitoring, that no animals suffered any injuries, became ill or had their survivorship negatively

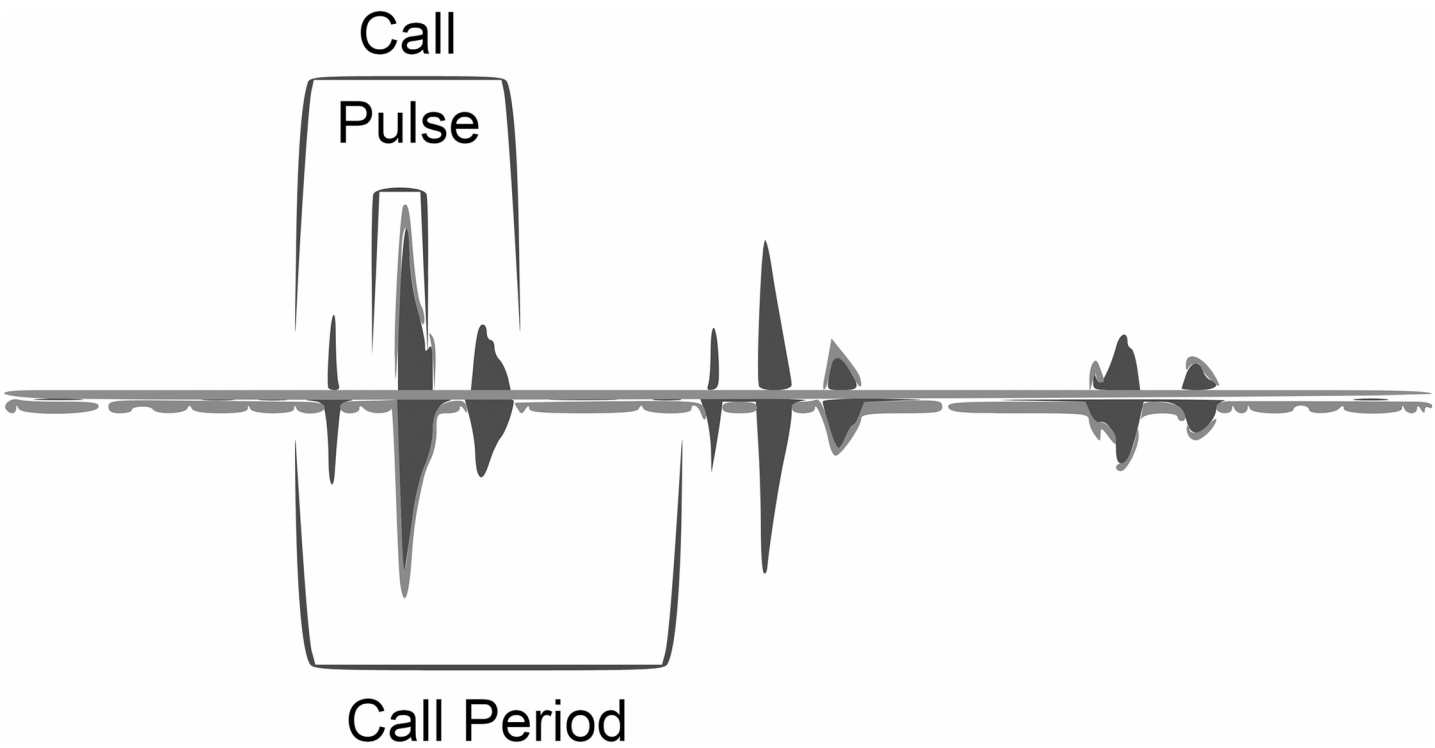

Fig 1. Wild golden mantella frog call waveform showing some measured call characteristics.

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affected as a result of this study. Furthermore we followed the Association for the Study of Animal Behaviour's Guidelines for the care of animals [18].

Recording calls

Frog calls were recorded using a digital audio recorder (H4n Handheld Digital Recorder, Zoom USA) with an omnidirectional microphone. Before recording calls, a pilot study was undertaken at the University of Manchester with their captive colony of golden mantella frogs to ensure the microphone and recorder had the appropriated sensitivity (i.e. could record all the frequencies emitted by the subjects). Recordings were analysed for call characteristics using Raven software [19]. The characteristics analysed were (Fig 1):

1. Call duration (s): Duration from the beginning of a call to its end.
2. Call period (s): Duration from the beginning of a call to the beginning of the next call.
3. Pulse rate: The number of individual components of each call.
4. Interpulse interval (s): Time between the pulses of a call.
5. Dominant frequency (Hz): The frequency with maximum intensity.

We analysed three call sequences of 20 different males *M*. *aurantiaca* from each population. In addition, to minimize intraspecific variance, we used mean values of the call parameters within and between individuals.

Phonotaxis experiments

Prior to any experimentation, measurements of sound pressure (noise) levels that animals are already exposed to during routine husbandry at Chester Zoo were taken using a sound pressure meter (SIP95 Sound Level Logging Meter FFT Audio Analyser, Balkon Technology) to avoid exposing animals to any extreme acoustic stimuli. Playback recordings were used with similar amplitude (i.e. volume) to what the animals were already exposed to in captive or natural environments. Calls were previously recorded from the three different populations using a digital audio recorder (H4n Handheld Digital Recorder, Zoom USA) with an omnidirectional microphone. Calls were edited for length and background noise using Audacity® [20] recording and editing software. During the experiment, we recorded the phonotaxis accuracy of a wild (Mangabe) and a captive population (Chester Zoo) of golden mantella frogs to three different recordings (used as treatments): one from a wild population of golden mantellas from Mangabe, and two from captive populations: one from Chester Zoo and one from Mitsinjo. Calls were presented using a randomized block design.

Active males were collected by hand from the ponds and put in a plastic box until the experiment. Frogs were kept in the box for nearly one hour, until they had recovered from being hand caught and were behaving normally with no signs of acute stress (i.e. abnormal behaviour, tachycardia). Each animal was tested only once. Phonotaxis playback experiments were than conducted by attracting *M. aurantiaca* males across a 100 x 60 cm PVC mat on the forest floor or enclosure floor to a Bluetooth speaker (model HX-P240PK, Jam Plus) broadcasting calls, similar to the method described by Mayer and colleagues (2014). During the experiment, 21 males from Chester zoo and 39 individuals from Mangabe had their phonotaxis response tested. Frogs were placed 10 cm away from the mat (see Fig 2). Trials were not scored if males did not enter the board from the front edge of the board. The experiment was videotaped with a Canon PowerShot SX520 HS digital camera.

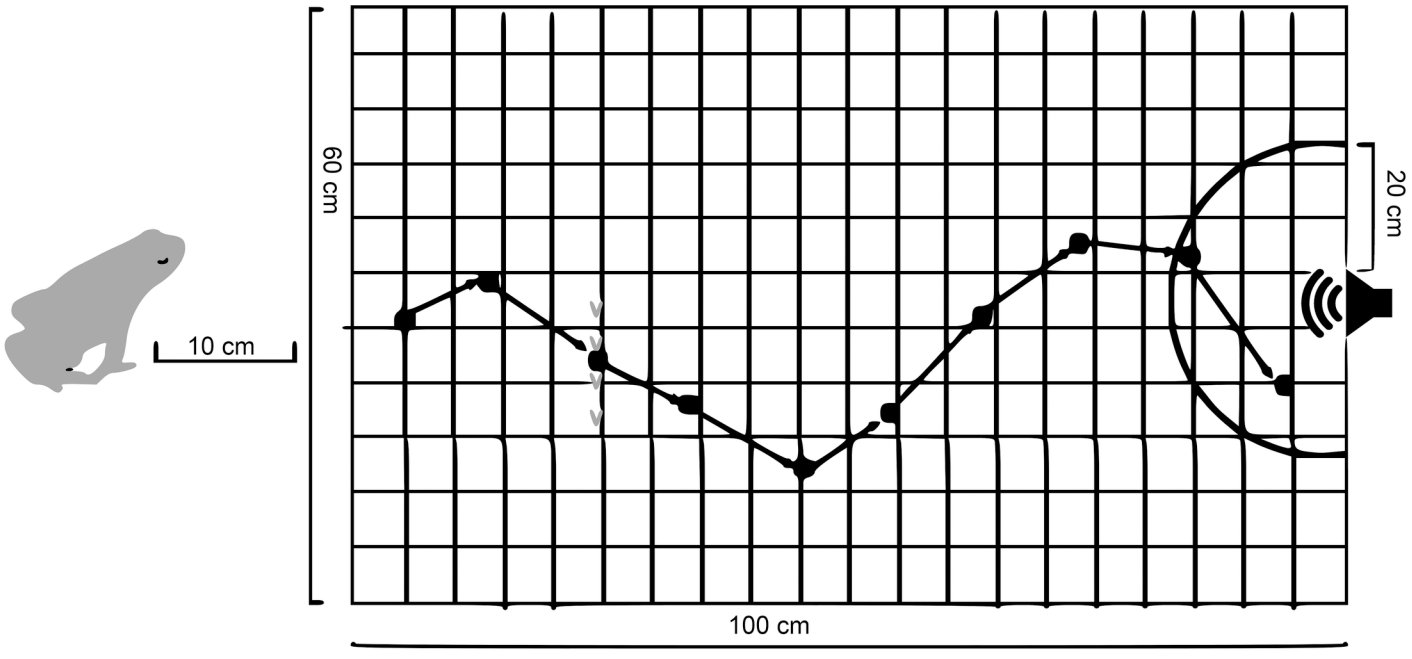

Fig 2. Schematic diagram of a male golden mantella frog when approaching a playback call on a speaker, the grid area is a PVC mat.

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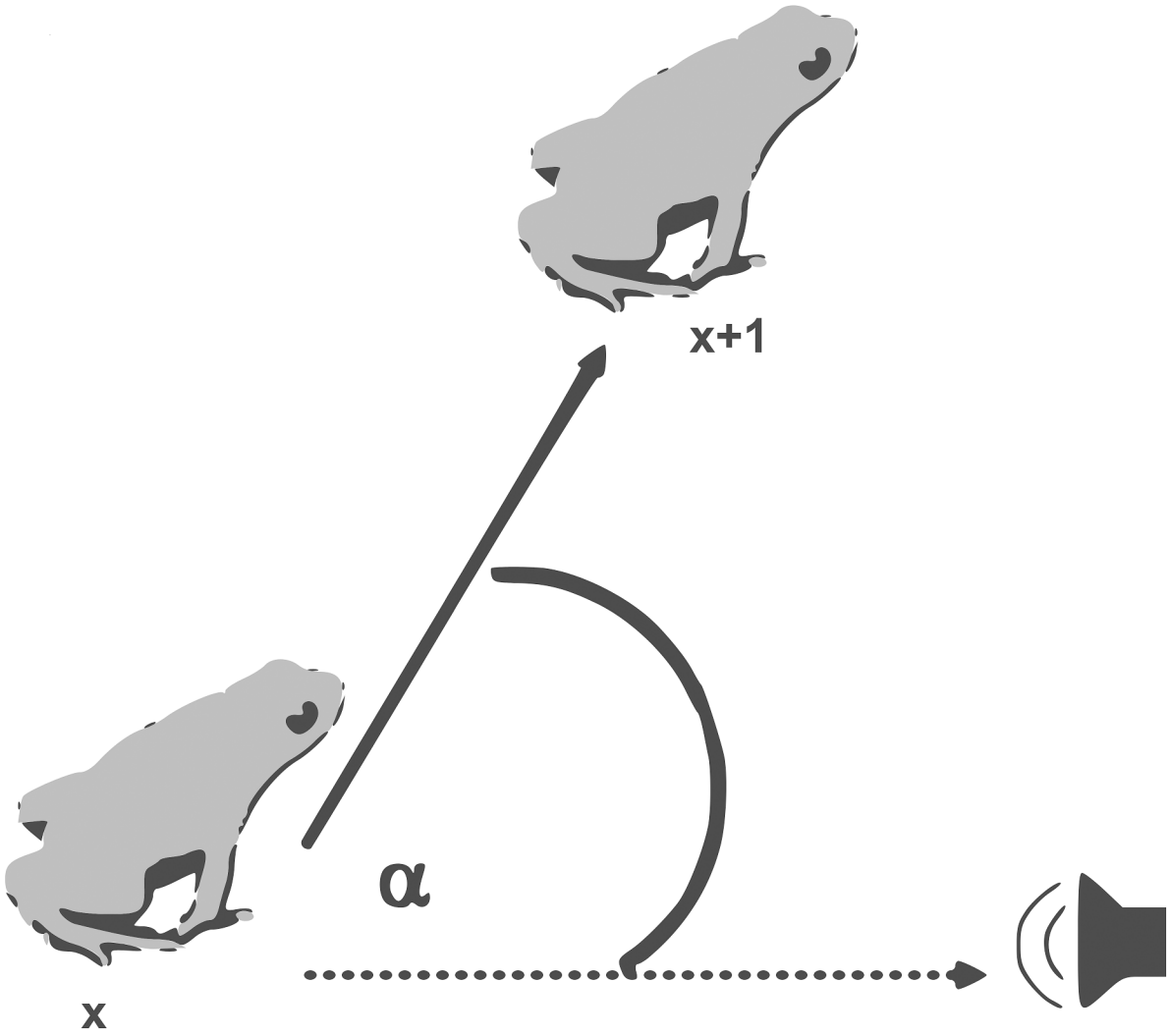

Fig 3. Illustration of how the jump angle α of male golden mantella frogs was calculated in a playback experiment. The dashed line indicates the straight line from the frog to the sound source, X the initial position of the frog and X + 1 the measured jump position.

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Previous playback studies with *Allobates femoralis* [11] and *Ranitomeya imitator* [13] revealed that at distances closer than 30 cm to the sound source the animals searched for a visual signal in addition to the acoustic stimulus; taking this in consideration, playback sessions ended when the frog reached within a perimeter of 20 cm of the speaker (Fig 2).

Movement analyses

Each jump of an approaching male was plotted by manually digitizing the recorded videos in a stop-motion view with software called BORIS [21]. The grid on the mat was used to identify frog positions and for calculating distances between positions and jump angles. Jump angles and distances were measured as soon as the animal had entered the board and until it came within 20 cm of the broadcasting speaker (Fig 2). For each playback, the following physical characteristics of frogs were analysed to define the accuracy of phonotaxis: (1) number of jumps; (2) jump angles (jump angle divergence of the new jump position to the target axis; Fig 3); (3) jump distances; (4)

path straightness (summing each jump distance for the path taken by the individual in relation to the straight line from the first entered position to the target); (5) duration (how long, in seconds, the frogs took to reach the speaker). The accuracy of the phonotactic approach was quantified using jump angles and the straightness of the path; values are given as percentage of path length in relation to the straight-line distance. All statistical analyses were done using R Studio [22].

Results

Call characteristics (Table 1) were compared between the three different populations using one-way ANOVA tests. Tests found significant differences between the populations on all the parameters analysed ($p<0.05$). The Tukey *posthoc* test (Table 2) confirmed that calls from Chester Zoo animals were significantly different ($p<0.05$ in all cases) from calls obtained from the wild population on all the analysed characteristics. Vocalisations from Mitsinjo breeding centre were significantly different from Mangabe calls in duration and period ($p<0.05$). Chester Zoo and Mitsinjo recording were statistically different in all parameters except for pulse numbers ($p<0.05$).

Phonotactic experiments resulted in 34 approaches of wild golden mantellas and 21 for the Chester Zoo's frogs (i.e. a total of 55 different individuals). In general, captive frogs took longer and used a lengthier and less accurate path to reach the speaker than wild frogs. All trials with Chester Zoo's frogs resulted in a phonotaxis response, however, five trials (two with Mitsinjo's calls, two with Chester's calls and one for Mangabe's calls) from Mangabe's animals, had no phonotaxis response (i.e. no movement) and were, therefore, not analysed. All successful trials were scored for number of jumps, jump distances, jump angles, path straightness and duration (Fig 4).

Generalised linear mixed models (GLMM) were used to compare the golden mantella frogs' phonotactic movement in response to different playback treatments (see Table 3). Calls were used as fixed factors and location as random factors. Wild individuals' responses to wild calls were used as the species' natural response and, this was considered as a reference for an expected reaction towards conspecifics. The wild frogs from Mangabe showed no difference ($p>0.05$) in any of the variables measured for all of the three calls (i.e., wild, or captive) used during the experiment.

Chester Zoo's frogs had significant differences ($p<0.05$) in the number of jumps and duration to the speaker when their own call was presented, jump angles for Mangabe and zoo calls, and path straightness between all calls (Table 4); however, different calls had no impact on jump distance ($p>0.05$). Despite frogs making a significantly higher number of jumps to reach the target, phonotaxis accuracy was higher for calls recorded at Chester Zoo with a straighter, shorter and faster path to the speaker (Fig 4). Path straightness when Mangabe's calls were played, resulted in a longer path in relationship to the path used during Chester Zoo calls, and an even longer path was used for Mitisinjo's playback calls.

Table 1. Call characteristics results for different wild and captive populations of golden mantella frogs.

Population	Origen	Duration (s)	Period (s)	Pulse rate	Interpulse (s)	Dominant frequency(Hz)
		± sd	± sd	± sd	± sd	± sd
Mangabe	Wild	0.043±0.004	0.09±0.05	2.92±0.27	0.008±0.002	4875±0.00
Chester Zoo	Captive	0.033 ±0.011	0.75±0.620	3.9±0.72	0.01±0.006	5198.01±172.84
Mitsinjo	Captive	0.062±0.008	0.12±0.063	4.04±0.19	0.005±0.001	4941.96±146.25

sd = standard deviation

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Table 2. *Posthoc* Tukey test results for golden mantella frogs' call characteristics from different wild and captive populations.

Populations	Duration	Period	Pulse rate	Interpulse	Dominant Frequency
Mangabe x Mitisnjo	p< 0.01	ns	p< 0.01	ns	ns
Mangabe x Chester	p< 0.01	p< 0.01	p< 0.01	p<0.05	p< 0.01
Mitisnjo x Chester	p< 0.01	p< 0.01	ns	p< 0.01	p< 0.01

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When the responses of both populations were compared using a t-test (Table 4) all the parameters were statistically different ($p<0.05$), except for jump distance. Wild frogs had a straighter, shorter and faster route even though they made shorter jumps (Fig 4).

Discussion

The analysis of different call parameters showed that calls from Chester Zoo's frogs were statistically different from wild frogs' vocalisations in all analysed characteristics. Whereas the call analyses from the colony held at Mitsinjo breeding centre showed greater similarities with the wild conspecifics. The implication of the observed differences could be negative in terms of reproduction if captive frogs were to be released to the wild. The breeding behaviour of golden mantella frogs involves males calling to court the females with multiple males vocalising simultaneously [23]. Males with calls modified by captivity, if reintroduced could have their ability to attract females compromised.

Vocalisations are moulded by the acoustic environment in which the species is found [24,25]. A zoo's environment has different background noises from sources such as heaters, air filters and visitors, which will lead to a different acoustic complexity (soundscape) than wild habitats. It has already been proved that anthropogenic sounds can alter the calling behaviour of anurans by causing males to modulate their call rate or call frequency [1,25]. Animals being kept in captivity for many generations could have their calls significantly affected by their environment, while frogs that have been in captivity for only one generation, would not be so affected. This would explain the results found on the call parameters of the Mitsinjo frogs, which had greater similarities with wild calls, while Chester Zoo animals had calls that were significantly different.

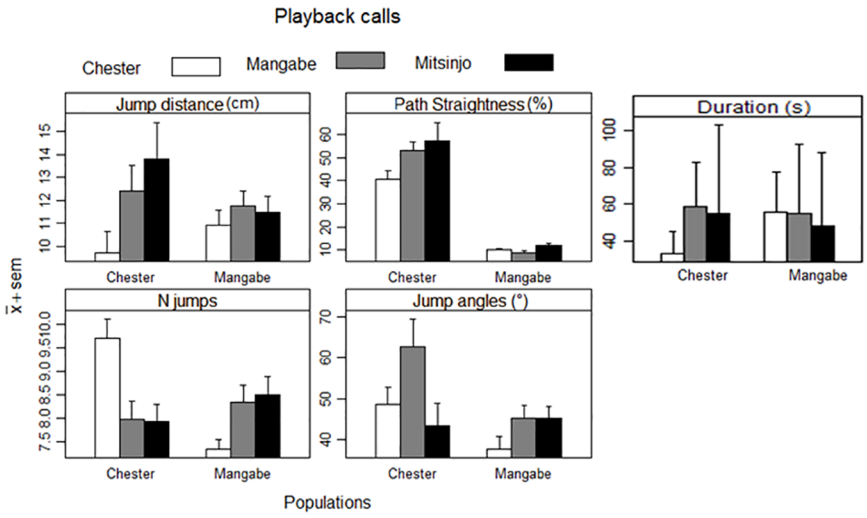

Fig 4. Summary of phonotactic movement results (mean +Standard Error of the Mean) of golden mantilla frogs towards playback calls.

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Table 3. Parameter estimates for the Generalized Linear Mixed Models describing the relationship between playback treatment (call sources) and analysis of phonotaxis response of male golden mantella frogs.

Population	Call	N	Parameter	Coefficient	p-value
Chester	Mangabe	7	N jumps	-0.04	ns
Chester	Mangabe		Jump angles	17.3	0.004
Chester	Mangabe		Jump distance	0.79	ns
Chester	Mangabe		Path straightness	39.9	0.006
Chester	Mangabe		Duration	10.59	ns
Chester	Mitsinjo	7	N jumps	-0.04	ns
Chester	Mitsinjo		Jump angles	-2.78	ns
Chester	Mitsinjo		Jump distance	2.29	ns
Chester	Mitsinjo		Path straightness	47.1	<0.001
Chester	Mitsinjo		Duration	6.39	ns
Chester	Chester	7	N jumps	0.09	<0.001
Chester	Chester		Jump angles	3.49	<0.001
Chester	Chester		Jump distance	-1.8	ns
Chester	Chester		Path straightness	32.2	0.024
Chester	Chester		Duration	7.08	<0.001
Mangabe	Mangabe	13	N jumps	-0.02	ns
Mangabe	Mangabe		Jump angles	1.27	ns
Mangabe	Mangabe		Jump distance	0.18	ns
Mangabe	Mangabe		Path straightness	-2.43	ns
Mangabe	Mangabe		Duration	6.43	ns
Mangabe	Mitsinjo	13	N jumps	2.15	ns
Mangabe	Mitsinjo		Jump angles	4.98	ns
Mangabe	Mitsinjo		Jump distance	1.53	ns
Mangabe	Mitsinjo		Path straightness	2.47	ns
Mangabe	Mitsinjo		Duration	7.8	ns
Mangabe	Chester	13	N jumps	-0.13	ns
Mangabe	Chester		Jump angles	1.27	ns
Mangabe	Chester		Jump distance	-0.58	ns
Mangabe	Chester		Path straightness	-2.73	ns
Mangabe	Chester		Duration	9.19	ns

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Table 4. T-test results of the movement analysis of phonotaxis response between wild and captive golden mantella frogs.

Location	Parameter	Mean	SEM	t	N	p-value
Wild	N jumps	8.04	0.18	1.97	55	0.02
Captive	N jumps	8.64	0.23			
Wild	Jump angles (˚)	51.79	3.17	2.54	55	0.04
Captive	Jump angles (˚)	42.62	1.72			
Wild	Jump distance (cm)	11.74	0.68	0.47	55	0.55
Captive	Jump distance (cm)	11.37	0.38			
Wild	Path straightness (%)	49.44	0.45	12.09	55	0.001
Captive	Path straightness (%)	10.33	2.99			
Wild	Duration (s)	49.18	2.00	3.15	55	0.001
Captive	Duration (s)	60.11	2.83			

https://doi.org/10.1371/journal.pone.0181931.t004

During the phonotaxis experiment we observed that wild frogs had a similar behavioural (phonotaxis) response to calls of conspecifics independent of their source (i.e. wild versus captive) while frogs from Chester Zoo had a significantly stronger response to their own calls. Wild frogs had more accurate response, reaching the speaker using a shorter path and in less time while captive frogs were using a longer path and more time, even though they had longer jumps. It is important to notice that wild frogs would recognize and react in a similar way to captive frogs despite the changes found in their calls. Captive frogs had a weak response to wild calls and, if captive frogs are not able to recognize wild calls or respond appropriately, this could, potentially have negative consequences[26, 27], such as assortative mating among released individuals, with females only being attracted to captive males, leading to two genetically disconnected populations [28]. This could, potentially, decrease the conservation value of the reintroduction programme.

The golden mantella frogs breeding behaviour is characterized by groups of males competitively calling to attract females; in this scenario it is usual to observe males showing aggressive behaviour toward other males as a sign of competition for females. This aggressive behaviour have been describe in the wild and observed in captive populations [28]. The phonotactic response observed in wild frogs corroborate with this premise, while captive frogs only showed this response to their own calls.

Species recognition is a fundamental problem for animals in social contexts [26] for a reintroduction to be successful, released individuals must survive and breed [27, 28].Although the accuracy of phonotaxis does not necessarily reflect the accuracy of perception, movement analysis is a powerful approach to examine the auditory abilities of animals [29]. When the responses of the two populations were compared, it was possible to observe that frogs from Mangabe (wild) showed a more precise phonotaxis response to calls than golden mantella frogs kept in captivity. Wild male golden mantella frogs would react to defend their territory against all possible opponents presented during the playback experiment, implying that they would recognize conspecific calls even from captive populations.

Animals in captivity are in a confined space in close proximity to other males [30], which could lead to overlapping territories and to recognition of individuals as neighbors and not as threats (i.e. "dear enemies"; [31]). This would explain the differences observed during the phonotaxis experiment, with captive animals using a longer and less accurate path and, taking longer to reach the speaker. Social recognition is thought to enhance fitness by providing a mechanism that allows animals to direct appropriate behaviours toward specific individuals during repeated social interactions, "the dear enemy effect" [32]. Evidence for the dear enemy effect typically consists of a relatively lower level of aggression exhibited by territory holders toward neighbours [32]. Dear enemy relationships, however, are not common among territorial species, and several studies have reported that territory residents respond similarly to neighbours and strangers under some conditions [31].

Frogs characteristically avoid moving unless totally necessary, since it is both energetically costly and increases predation risk [4]. The receiver of an acoustic signal has to judge the sender's motivational state and adjust his own reaction according to the costs [29]. If calls are not perceived as intruders, but as neighbours, it would not trigger such a phonotaxis response. The decision to approach and chase an intruder is, therefore, influenced by the trade-off between fitness costs and benefits [29].

Animals may adjust the characteristics of their vocalizations in response to temporary changes in the background noise [23,1]. Such short-term vocal adaptations have been examined in insects, anurans, birds, and mammals [1]. Pre-release training associated with a soft release programme, could help re-shape calls from captive animals to increase their chances of

breeding in the wild. Similar approaches have been used successfully in golden lion tamarins (*Leontopithecus rosalia*) [31].

Communication can be crucial for breeding success in golden mantella frogs if individuals are being bred for conservation; it is of critical importance to make sure that captive animals, if released, will have the same chances of breeding as their wild counterparts. Captive breeding is growing as an indispensable tool in conservation tool for many species [33], especially amphibians. However, it is important to fully understand the impact of captivity on a species' behaviour before releasing individuals back into the wild.

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PLOS ONE

RESEARCH ARTICLE

The tonic immobility test: Do wild and captive golden mantella frogs (*Mantella aurantiaca*) have the same response?

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Abstract

Adaptations to captivity that reduce fitness are one of many reasons, which explain the low success rate of reintroductions. One way of testing this hypothesis is to compare an important behavioural response in captive and wild members of the same species. Thanatosis, is an anti-predator strategy that reduces the risk of death from predation, which is a common behavioral response in frogs. The study subjects for this investigation were captive and wild populations of *Mantella aurantiaca*. Thanatosis reaction was measured using the Tonic Immobility (TI) test, a method that consists of placing a frog on its back, restraining it in this position for a short period of time and then releasing it and measuring how much time was spent feigning death. To understand the pattern of reaction time, morphometric data were also collected as body condition can affect the duration of thanatosis. The significantly different TI times found in this study, one captive population with shorter responses, were principally an effect of body condition rather than being a result of rearing environment. However, this does not mean that we can always dismiss the importance of rearing environment in terms of behavioural skills expressed.

Introduction

Considerable difficulty has been encountered in successfully reintroducing endangered species into their natural habitats, and adaptations to captivity that reduce fitness in the wild (e.g. lack of predator recognition and appropriate response) are one of several reasons for this low success rate [1]. If captive animals are to be released into the wild, these issues should be addressed [2]. Evaluating the behavioural skills of captive bred animals could allow the selection of appropriate individuals and lead to improvements in the success rates of reintroduction programs [3]. This has been showed for different species such as black-footed ferrets (*Mustela nigripes*) [3], Caribbean rock iguanas (*Cyclura sp.*) [4] and different fish species [5].

One of the most important responses to preserve in captive populations destined for reintroduction is the ability to detect and respond appropriately to natural predators [4,6]. It is known that captivity can cause animals to: lose natural responses, have insufficient fear of humans, and express abnormal behaviour [5,7,8]. These can limit the success of subsequent

reintroduction attempts [5,7,8]. An example is the first attempts to release golden lions tamarins *(Leontopithecus rosalia)*, that failed due the lack of behavioral skills possessed by captive reared individuals [3]. Captive environments are often highly predictable and without threatening stimuli, this could lead to important anti-predator responses being weakened or even disappearing during generations of captive breeding [5,6,9].

Tonic immobility (TI), or thanatosis, is behavioural motor inhibition and reduced responsiveness to external stimulation induced by physical restraint [10]. The TI response is considered as an adaptive behavioural anti-predator strategy, reducing the threat of death from predation and, thereby, increasing the chances of survival [11]. While displaying thanatosis an animal adopts a posture that gives it the appearance of being dead with which it may inhibit or divert the attack of a potential predator [11]. Toxic animals, such as golden mantella frogs, display conspicuous body coloration, and their immobile posture would often enhance the effectiveness of aposematism [12]. Tonic immobility could induce the predator to loosen its hold on the prey, thereby providing a chance of escape [11,13].

Tonic Immobility has been documented as a behaviour expressed by a wide variety of species including mammals, insects, reptiles, birds, fish and amphibians [10,11,13,14,15,16]. This response seems specific to threatening situations; the more intense the stimulus is, the longer the TI response is [11]. It is known that different factors can influence thanatosis duration such as stress levels [17], welfare status [13], stimulus intensity [18], predation pressure [19] and environmental disturbances [20] amongst others. Studies with frog species have demonstrated that stressful stimuli such as loud noises (*Rana pipiens*, [20]), extreme temperatures (*Rana temporaria*, [21]) or the sight of predators (*Platymantis vitiana*, [18]) can affect TI response duration of captive animals.

It is crucial to conserve the behavioural integrity of captive wildlife, particularly if animals are to be used for conservation efforts including reintroductions [22,23]. Therefore, investigations as to whether captive breeding centres are providing the stimuli to allow species to fully develop their behavioral repertoire are crucial [23]. The aim of this study was to compare tonic immobility responses of wild and captive golden mantella frogs (*Mantella aurantiaca)*, thereby assessing the effects of captivity on this survival strategy. As death feigning is a natural defensive response [11, 14, 18] it was predicted that wild frogs will have a longer TI response since these individuals are expected to be more experienced in expressing defensive behaviours due to the threats in their habitat. Captive bred animals can be naive to the threat of predation and, therefore, might be unable to generate adequate physiological and behavioural responses to a threatening stimulus [18]. Tonic immobility is also associated with fear [18], since captive frogs are also habituated to handling and human interaction (e.g. during cleaning and feeding routines): a human interaction should not trigger such a fear response [24].

Methodology

Ethical approval

All the research reported in this study was approved by the Ethics Commission of Chester Zoo, UK and it conforms to all regulations and laws in all relevant countries in relation to care of experimental animal subjects. Furthermore we can confirm, from our post-experimental monitoring, that no animals suffered any injuries, became ill or had their survivorship negatively affected as a result of this study.

Study subjects

The model species for this study was the golden mantella frog (*M. aurantiaca).* It is a species classified as critically endangered by the IUCN [25] and is endemic to the Moramanga district, in the

Region of Alaotra-Mangoro, Madagascar. It is well known due to its aposematic orange-red colouration and presence in the international pet trade [25]. Potential predators for the species would be reptile species such as *Zonosaurus madagascariensis* and *Tamnosophis lateralis* [26]. Its distribution is restricted to a fragment of humid forest around seasonally flooded ponds surrounded by degraded land [25]. A significant proportion of its population is located inside or near the area of the Ambatovy mine [27]. Following a conservation needs assessment, the Amphibian Ark prioritised *M. aurantiaca* as a species in need of *ex situ* assistance to safeguard its survival [27,28,29].

Study sites

Mangabe area. Mangabe rainforest is a site of international biodiversity importance, being home to almost half of the world's breeding ponds for the golden mantella frog according to recent studies on high conservation priority sites for mantella frogs. Mangabe forest, or the 'blue forest', covers approximately 40,000 ha in eastern Madagascar and is divided between two administrative districts, Moramanga in the north and Anosibe An'ala to the south. Data sampling for this study was done in the Moramanga region. The data from wild frogs (N = 90) at Mangabe were obtained during October 2014 and again in February 2015.

Ambatovy mining site. Ambatovy's mine is located within a species-rich region of Madagascar at the southern end of the remaining Eastern Forest Corridor in the Moramanga region. As part of the Environmental Management Plan, there is a Conservation Zone of native forest maintained by the mining company. Pre-clearance species inventories and translocation of live animals to conservation forest refuge areas called the Receptor Ponds were carried by Madagasikara Voakajy, a local NGO involved in the conservation of golden mantellas. During this study, animals from the Conservation Zone and animals that were translocated to Receptor ponds were sampled. Ambatovy population (N = 30) was sampled in March 2016.

Chester Zoo, UK. Chester Zoo is actively involved in the conservation of the golden mantella frogs in Madagascar. The zoo currently maintains two *ex situ* groups of *M. aurantiaca*, one is on public display at the Zoo's Tropical Realm exhibit and a second group is kept off show in a biosecurity container specifically for conservation-related research. Frogs are kept in naturalistic tanks with different live species of plants, moss for substrate, water, hiding places under rocks, UV light and heaters to mimic the natural conditions found in Madagascar. Animals are fed different live invertebrates with diet supplementation. The Chester Zoo population (N = 30) was sampled in March 2016.

Mitsinjo Association Captive Breeding Centre. Mitsinjo Association is a community-run conservation organization. This is Madagascar's first biosecure facility to safeguard amphibians from extinction, and currently maintains a genetically viable population of the golden mantella frog taken from the Ambatovy mining site (i.e., genetic founders). The offspring (F1) of these individuals are intended for reintroductions at artificially created breeding and natural ponds. Animals are kept in tanks with aquarium gravel as substrate, a plant pot, water, coconut shells for hiding. No UV light was supplied. Animals were fed a variety of live invertebrates, but no food supplementation is given. During this project, only data from the founders' offspring (F1) were collected. The data from the captive frogs from the Mitsinjo captive breeding centre (N = 20) were obtained in February 2015.

TI test

Thanatosis reaction was measured using the Tonic Immobility (TI) test, a standardised method that consistently and reliably induces TI [10,13]. Frogs were caught and immediately subjected to the TI test (within 3 s). Each individual was placed on its back in the palm of the experimenter's hand and restrained in that position for 10 s using gentle pressure on its belly

from the experimenter's thumb, and then released. If a frog moved 3 s after release, then it was considered that TI had not been induced. In this case, the restraint was repeated up to three times. If TI was not induced after 3 attempts, a score of 0 s was given. Conversely, if frogs did not show any movement after 5 min, the test was terminated and a maximum score of 300 s was given for tonic immobility duration. Animals were always handled by the same researcher. Tonic immobility can be affected by ambient temperature [15,21], Chester Zoo facilities are kept in a temperature controlled environment to mimic Madagascar climate conditions. Mitsinjo facilities' temperature is allowed to fluctuate with the climate outside since the captive population was maintained within the native range of the species [25]. For this reason temperature was not used as a possible source of variation (i.e. factor).

Body condition index

Body condition index (BCI) was assessed using the Scaled Mass Index proposed by Peig and Green [30]. This method is independent of size and can be used for comparison between different populations; these characteristics potentially make it superior to the traditional residual indices and, reportedly it has worked well in amphibian studies [31,32]. The scaled mass index of condition (M_i) was calculated as follows:

$$M_i = M * \left[\frac{SVL_o}{SVL}\right]^{bSMA}$$

where M and SVL are the mass and Snout-vent length of the individual, SVL_0 is the arithmetic mean SVL of the population, and bSMA is the standardized major axis slope from the regression of ln M on ln SVL for the population [30]. Each individual was measured (±0.01mm) for SVL using a digital calliper (Lujii 150mm, Omiky) and body mass was obtained using a precision scale (accurate to 0.01g, Smart Weigh ACC200 AccuStar).

Data analysis

Data were confirmed to have a normal distribution using the Shapiro-Wilk normality test. There were no statistical differences between BCI and TI responses between the two sample periods in Mangabe, and between the two populations from Chester Zoo, for this reason, data were analysed together. TI responses and BCI were compared using ANOVA tests. A Pearson correlation was used to analyse BCI and TI responses. Statistical analyses were done using R Studio [33].

Results

There was no significant difference in TI responses among groups (wild and captive) ($F = 1.901$, $df = 1$, $p = 0.17$), but there was a significant difference between populations ($F = 12.23$, $df = 4$, $p<0.001$). The Tukey *post-hoc* analyses showed that the golden mantella frog population kept at Mitsinjo Breeding Centre had a significantly ($p<0.01$) shorter duration TI response when compared to all other groups (Table 1) and no other significant differences were detected.

After obtaining a body condition index for all individuals (Table 2), groups (wild x captive) were compared using a one-way ANOVA test ($F = 8.278$, $df = 1$, $p = ns$). The test showed that there was no significant difference between groups.

There was no significant difference on the body condition index between groups (wild and captive ($F = 0.569$, $df = 1$, $p = 0.45$) and a significant difference between populations ($F = 9.289$, $df = 4$, $p<0.001$). The Tukey *post-hoc* analyses confirmed that animals from Mitsinjo were significantly different from all other groups with a much lower body condition.

Table 1. Tonic immobility test results for different wild and captive populations of golden mantella frogs.

Population	Group	N	Max (secs)	Min (secs)	Mean (secs)	St. Dev (secs)
Mangabe	Wild	90	180	0	78.54	47.40
Ambatovy—Receptor	Wild	30	147	0	81.00	67.00
Ambatovy -Conservation	Wild	30	180	0	71.31	59.06
Mitsinjo Breeding Centre	Captive	20	40	0	10.05	13.72
Chester Zoo	Captive	30	136	30	83.63	29.99

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A significant positive correlation was found between TI responses and body condition index scores when all data were compared using a Pearson correlation test ($r = 0.02$, $N = 200$, $p<0.05$.; observation: 4 outliers removed ($r = 0.33$, $N = 196$, $p<0.001$), which had very large standardised residuals) and when each population was analysed separately (Table 3, Fig 1). Animals with better body condition had longer responses.

Discussion

In this study we showed that wild populations of golden mantella frogs and those kept at Chester Zoo had similar TI response durations, whereas animals kept at Mitsinjo breeding centre had a significantly shorter TI duration. These results suggest that captivity is not the only factor involved in the shorter durations observed in one of the captive colonies. Animals from Chester Zoo, which have been in captivity for many more generations, still presented the same response as the wild populations. On the other hand, frogs kept at Mitsinjo breeding centre after the first generation in captivity presented a shorter response when compared to wild animals. This is true even when compared to the wild population from where their parental generation were collected, which also discounts the results being due to some natural variation between populations.

During this study, there was also a significant difference in the body condition of animals between the populations. Body condition is a valuable index that can be assessed using reliable, non-invasive techniques, and it can identify the health condition of a population before any deleterious effects can be observed [31]. The data collected from wild and captive *M. aurantiaca* showed that the individuals kept at the Mitsinjo breeding centre had a much lower body condition index than any other group. Again, this cannot be generalized as a consequence of captivity, since frogs from Chester Zoo present no statistical difference on BCI when compared to the wild populations. This result could be used to infer that animals at Mitsinjo are not in ideal health condition when compared with other analysed populations.

Lower body condition could be a result of different factors such as diet, reproductive stage and age [34]. Both captive colonies receive a diet of variety of live invertebrates, but Chester Zoo's colony also received a diet supplementation. There is a lack of knowledge concerning

Table 2. Body condition index score results for different wild and captive populations of golden mantella frogs.

Population	Group	N	Max	Min	Mean	St. deviation
Mangabe	Wild	90	1.54	0.42	0.89	0.16
Ambatovy—Receptor	Wild	30	2.29	0.56	0.88	0.40
Ambatovy -Conservation	Wild	30	1.01	0.49	0.87	0.11
Mitsinjo Breeding Centre	Captive	20	1.28	0.39	0.67	0.19
Chester Zoo	Captive	30	1.12	0.40	0.91	0.32

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Table 3. Pearson correlation results for relationship between tonic immobility response (duration) and body condition index for different golden mantella frog populations.

Population	r	N	p-Value
Mangabe	0.06	90	<0.05
Ambatovy—Receptor	0.07	29	<0.05
Ambatovy -Conservation	0.15	29	<0.01
Mitsinjo Breeding Centre	0.06	19	<0.05
Chester Zoo	0.04	29	<0.01

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the nutritional necessities and absorption efficiency of amphibians; however, studies have demonstrated that diet supplementation can have a positive impact on frog body condition and general health [35]. This lack of vitamin and mineral supplementation could be causing frogs from Mitsinjo to have a lower body condition.

There is also a reported relationship between weight-loss and stress in captive individuals [17,34]. Captivity can present many sources of stress, possibly the greatest stressors are those over which the animal has no control and from which they cannot escape, such as a poor diet, inadequate habitat and restricted movement [17]. Chronic stress may be indicated by a wide range of physiological responses including inhibited growth rate [36,37], reduced body weight [38,39], and reduced food intake [40]. Persistent exposure to continuous stressors can have

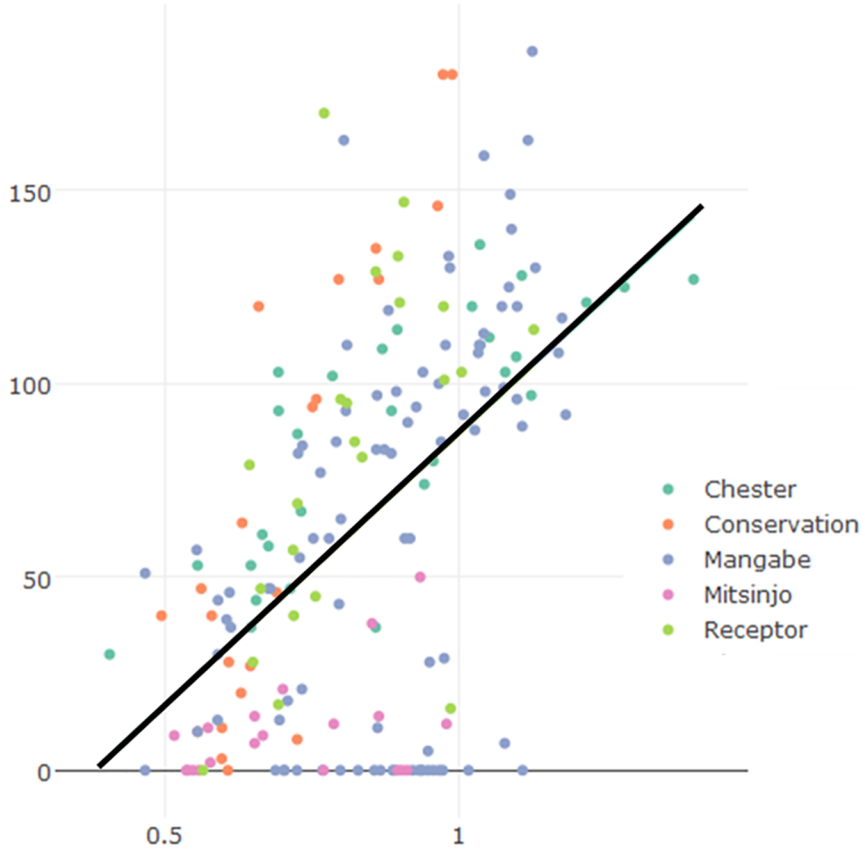

Fig 1. Scatter plot of body condition index (BCI) and tonic immobility (TI) response (s) of different populations of golden mantella frogs.

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many deleterious consequences for captive animals putting at risk the long-term health of captive animals [23,36,41,42,43,44]. Environmental factors, such as providing the correct UV light standards, could be involved in maintaining the healthy state of frogs kept in captivity [32,45,46] The lack of UV light provision for the Mitsinjo colony could, also, be involved at the low body condition.

The positive correlation between TI response and BCI showed that body condition was an important factor in the duration of the tonic immobility response; individuals with lower body condition had shorter responses independent of origin. Even though a correlation was found it is important to state that it was a weak correlation. Possibly other factors are involved in the TI responses. The results found here showed that husbandry differences, and not just being in captivity per se, had an impact on the health conditions of frogs and as a consequence affected their behavioural responses.

TI response is an acute stress response to a short term elevation of corticosterone levels, as has already been demonstrated in experiments using Fijian ground frogs (*Platymantis vitiana)* [18]. A short term elevation of stress hormones could be caused by a predator attack or the simulation of one (Tonic immobility test). A short-term increase in the corticosterone levels can promote key changes in the behaviour and physiology that enables individuals to cope with stress [19]: an acute stress response. Some of the key behaviours affected by corticosterone in amphibians are defensive behaviours such as tonic immobility [18]. However, if frogs from Mitsinjo were already experiencing chronic levels of stress due to a poor diet and environment, it is possible that their acute stress responses could be blunted [46], such as TI responses.

Body condition index can be used to assess the chronic levels of stress of captive animals [41], while TI response could be an alternative technique to asses acute stress responses on captive individuals. The stress response is not inherently detrimental, but rather, is a complex and essential negative-feedback process [47]. The capacity to cope with threatening (acute stress) situations is a vital ability to survival in the wild [35]. Predation, competition and other stressful events are part of the routine in the wild habitats.

A biosecurity facility for the conservation of amphibians on site is very important step for the future of many different species [48]. However, maintaining the necessary standards to keep animals fit for reintroductions is still a challenge. The husbandry differences, provision of UV light and diet supplementation, found between Chester Zoo and Mitsinjo reflect the availability of equipment and diet supplements in each country. Reintroductions are costly and time consuming; therefore, to make the best use of resources available it is important to screen individuals that are destined for reintroduction.

Captive environments are different from the wild and can impose different selection pressures or relaxed selection pressures leading to adaptation to captivity and, consequently, affecting behaviour including anti-predators responses [1,8,21,48]. The importance of maintaining the behavioural integrity of zoo populations, especially those that are used for conservation efforts including reintroductions is critical for the conservation of biodiversity [21]. Amphibians have long been neglected in research into animal welfare and behavioural problems related to captivity; this is clear in the historic lack of enriched captive environments to encourage natural behaviour and psychological well-being [48].

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Appendix 1. List of all Operational Taxonomic Units (OTUs) identified during the 16S Next Generation Sequencing in each of the sample populations (Mangabe (wild), Ambatovy (Wild) and Chester Zoo (captive)).

OTUid	Kingdom	Phylum	Class	Order	Family	Genus	Mangabe	Ambatovy	Chester
OTU_1	Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Serratia	*	*	*
OTU_2	Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	NA	*	*	*
OTU_3	Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Salmonella	*	*	*
OTU_4	Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Serratia	*	*	*
OTU_5	Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Enterobacter	*		*
OTU_6	Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Microbacteriaceae	NA	*	*	*
OTU_7	Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Providencia	*		*
OTU_8	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Alcaligenaceae	Bordetella	*		*
OTU_9	Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	NA			*
OTU_10	Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Proteus			*
OTU_11	Bacteria	NA	NA	NA	NA	NA	*		
OTU_12	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Bradyrhizobiaceae	Bradyrhizobium	*		
OTU_13	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	Enhydrobacter		*	*
OTU_14	Bacteria	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	Rhodanobacter			*
OTU_15	Bacteria	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Sphingomonas	*		
OTU_16	Bacteria	Cyanobacteria	Chloroplast	Chloroplast	Bacillariophyta	NA			*
OTU_17	Bacteria	Proteobacteria	Gammaproteobacteria	Oceanospirillales	Halomonadaceae	Halomonas			*
OTU_18	Bacteria	Saccharibacteria	NA	NA	NA	NA			*
OTU_19	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas		*	*
OTU_20	Bacteria	Proteobacteria	Betaproteobacteria	Methylophilales	Methylophilaceae	Methylophilus			*
OTU_21	Bacteria	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Sinobacteraceae	Alkanibacter			*
OTU_22	Bacteria	Cyanobacteria	Chloroplast	Chloroplast	Streptophyta	NA	*		*
OTU_23	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	NA	NA		*	
OTU_24	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Beijerinckiaceae	NA		*	
OTU_25	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Alcaligenaceae	Candidimonas			*
OTU_26	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	NA	NA	*		
OTU_27	Bacteria	Proteobacteria	Gammaproteobacteria	Oceanospirillales	Alcanivoracaceae	Alcanivorax			*
OTU_28	Bacteria	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	Wohlfahrtiimonas			*
OTU_29	Bacteria	Proteobacteria	Betaproteobacteria	Methylophilales	Methylophilaceae	Methylophilus			*
OTU_30	Bacteria	Proteobacteria	Alphaproteobacteria	Alphaproteobacteria_incerta	Rhizomicrobium	NA	*		

				e_sedis					
OTU_31	Bacteria	Actinobacteria	Actinobacteria	Rubrobacterales	Rubrobacteraceae	Rubrobacter	*		
OTU_32	Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Brevibacteriaceae	Brevibacterium		*	*
OTU_33	Bacteria	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Sinobacteraceae	Alkanibacter			*
OTU_34	Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Propionibacteriaceae	Propionibacterium	*	*	
OTU_35	Bacteria	Proteobacteria	NA	NA	NA	NA			*
OTU_36	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Burkholderiaceae	Burkholderia	*	*	
OTU_37	Bacteria	Saccharibacteria	NA	NA	NA	NA		*	
OTU_38	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas		*	
OTU_39	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	Alkanindiges		*	
OTU_40	Bacteria	Firmicutes	Bacilli	Bacillales	Staphylococcaceae	Staphylococcus	*	*	
OTU_41	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Methylobacteriaceae	Methylobacterium	*	*	
OTU_42	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	NA	NA			*
OTU_43	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Beijerinckiaceae	NA	*		
OTU_44	Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Microbacteriaceae	Microbacterium		*	
OTU_45	Bacteria	Acidobacteria	Acidobacteria_Gp4	Blastocatella	NA	NA		*	
OTU_46	Bacteria	Actinobacteria	Actinobacteria	Solirubrobacterales	Conexibacteraceae	Conexibacter	*		
OTU_47	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Aurantimonadaceae	Aurantimonas			*
OTU_48	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Beijerinckiaceae	Beijerinckia	*		
OTU_49	Bacteria	Planctomycetes	Planctomycetia	Planctomycetales	Planctomycetaceae	Singulisphaera	*		
OTU_50	Bacteria	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	NA			*
OTU_51	Bacteria	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	Luteibacter			*
OTU_52	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Acidovorax	*		
OTU_53	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Burkholderiaceae	Burkholderia	*		
OTU_54	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Rhizobacter	*	*	
OTU_55	Bacteria	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	Arenimonas	*		
OTU_56	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Variovorax	*		
OTU_57	Bacteria	Verrucomicrobia	Spartobacteria	NA	NA	NA		*	
OTU_58	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Bradyrhizobiaceae	Bradyrhizobium		*	
OTU_59	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	*		
OTU_60	Bacteria	Acidobacteria	Acidobacteria_Gp2	Gp2	NA	NA	*	*	
OTU_61	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	NA	*		

OTU_62	Bacteria	Proteobacteria	Alphaproteobacteria	Rhodospirillales	NA	NA		*	
OTU_63	Bacteria	Actinobacteria	Actinobacteria	Acidimicrobiales	Iamiaceae	Iamia	*	*	
OTU_64	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	NA		*	
OTU_65	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	NA	NA		*	
OTU_66	Bacteria	Acidobacteria	Acidobacteria_Gp1	Gp1	NA	NA		*	
OTU_67	Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Pseudonocardiaceae	Pseudonocardia			*
OTU_68	Bacteria	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	Rhodanobacter			*
OTU_69	Bacteria	Verrucomicrobia	Spartobacteria	NA	NA	NA		*	
OTU_70	Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Micrococcaceae	Micrococcus		*	
OTU_71	Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Dermacoccaceae	NA			*
OTU_72	Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Mycobacteriaceae	Mycobacterium		*	
OTU_73	Bacteria	Planctomycetes	Planctomycetia	Planctomycetales	Planctomycetaceae	Aquisphaera		*	
OTU_74	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Myroides			*
OTU_75	Bacteria	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Sphingobacteriaceae	Mucilaginibacter	*		
OTU_76	Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Nocardioidaceae	Aeromicrobium		*	
OTU_77	Bacteria	NA	NA	NA	NA	NA		*	
OTU_78	Bacteria	Acidobacteria	Acidobacteria_Gp1	Gp1	NA	NA		*	
OTU_79	Bacteria	Acidobacteria	Acidobacteria_Gp1	Acidipila	NA	NA		*	
OTU_80	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	Acinetobacter		*	
OTU_81	Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Micromonosporaceae	Actinoplanes	*		
OTU_82	Bacteria	Proteobacteria	Epsilonproteobacteria	Campylobacterales	Helicobacteraceae	Helicobacter		*	
OTU_83	Bacteria	Verrucomicrobia	Spartobacteria	NA	NA	NA		*	
OTU_84	Bacteria	Planctomycetes	Planctomycetia	Planctomycetales	Planctomycetaceae	Aquisphaera		*	
OTU_85	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Oxalobacteraceae	Janthinobacterium		*	
OTU_86	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	NA		*	
OTU_87	Bacteria	Proteobacteria	Gammaproteobacteria	Oceanospirillales	Alcanivoracaceae	Alcanivorax			*
OTU_88	Bacteria	Acidobacteria	Acidobacteria_Gp2	Gp2	NA	NA	*	*	
OTU_89	Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Mycobacteriaceae	Mycobacterium			*
OTU_90	Bacteria	Acidobacteria	Acidobacteria_Gp1	Gp1	NA	NA	*		
OTU_91	Bacteria	Planctomycetes	Planctomycetia	Planctomycetales	Planctomycetaceae	Aquisphaera		*	
OTU_92	Bacteria	Deinococcus-Thermus	Deinococci	Deinococcales	Deinococcaceae	Deinococcus		*	
OTU_93	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Burkholderiaceae	Burkholderia		*	

OTU_94	Bacteria	Proteobacteria	NA	NA	NA	NA	*		
OTU_95	Bacteria	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Chitinophagaceae	NA	*		
OTU_96	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Beijerinckiaceae	Methylovirgula	*		
OTU_97	Bacteria	Acidobacteria	Acidobacteria_Gp1	Granulicella	NA	NA	*		
OTU_98	Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Corynebacteriaceae	Corynebacterium		*	
OTU_99	Bacteria	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Sinobacteraceae	Alkanibacter			*
OTU_100	Bacteria	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	Luteibacter			*
OTU_101	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Cryomorphaceae	Fluviicola	*		
OTU_102	Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Mycobacteriaceae	Mycobacterium		*	
OTU_103	Bacteria	Planctomycetes	Planctomycetia	Planctomycetales	Planctomycetaceae	NA		*	
OTU_104	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Burkholderiaceae	Ralstonia		*	
OTU_105	Bacteria	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Chitinophagaceae	Flavisolibacter		*	
OTU_106	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	Rhizobium		*	
OTU_107	Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Corynebacteriaceae	Corynebacterium		*	
OTU_108	Bacteria	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Sphingomonas			*
OTU_109	Bacteria	Firmicutes	Bacilli	Lactobacillales	Enterococcaceae	Vagococcus			*
OTU_110	Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	NA	NA			*
OTU_111	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Hyphomicrobiaceae	Devosia	*		
OTU_112	Bacteria	Verrucomicrobia	Spartobacteria	NA	NA	NA	*		
OTU_113	Bacteria	NA	NA	NA	NA	NA		*	
OTU_114	Bacteria	Proteobacteria	Gammaproteobacteria	NA	NA	NA		*	
OTU_115	Bacteria	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	Luteibacter			*
OTU_116	Bacteria	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Sphingomonas		*	
OTU_117	Bacteria	Verrucomicrobia	Subdivision3	NA	NA	NA		*	
OTU_118	Bacteria	NA	NA	NA	NA	NA		*	
OTU_119	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	Acinetobacter		*	
OTU_120	Bacteria	NA	NA	NA	NA	NA		*	
OTU_121	Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Mycobacteriaceae	Mycobacterium	*		
OTU_122	Bacteria	Acidobacteria	Acidobacteria_Gp1	Granulicella	NA	NA	*		
OTU_123	Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Citrobacter	*		
OTU_124	Bacteria	Bacteroidetes	Cytophagia	Cytophagales	Cytophagaceae	Spirosoma		*	
OTU_125	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Chryseobacterium			*
OTU_126	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Hyphomicrobiaceae	Devosia	*		

OTU_127	Bacteria	Planctomycetes	Planctomycetia	Planctomycetales	Planctomycetaceae	Planctomyces	*		
OTU_128	NA	NA	NA	NA	NA	NA	*		
OTU_129	Bacteria	Acidobacteria	Acidobacteria_Gp1	Gp1	NA	NA		*	
OTU_130	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	NA	NA		*	
OTU_131	Bacteria	Acidobacteria	Acidobacteria_Gp1	Gp1	NA	NA		*	
OTU_132	Bacteria	Proteobacteria	Deltaproteobacteria	Myxococcales	NA	NA		*	
OTU_133	Bacteria	Firmicutes	Bacilli	Bacillales	Bacillaceae_1	Bacillus		*	
OTU_134	Bacteria	Actinobacteria	Actinobacteria	Solirubrobacterales	Conexibacteraceae	Conexibacter			*
OTU_135	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	*		
OTU_136	Bacteria	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Sphingobacteriaceae	Sphingobacterium		*	
OTU_137	Bacteria	candidate_divisio	NA	NA	NA	NA		*	
OTU_138	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Burkholderiaceae	Burkholderia		*	
OTU_139	Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Dermacoccaceae	Branchiibius		*	
OTU_140	Bacteria	Verrucomicrobia	Subdivision3	NA	NA	NA		*	
OTU_141	Bacteria	NA	NA	NA	NA	NA		*	
OTU_142	Bacteria	Acidobacteria	Acidobacteria_Gp1	Acidobacterium	NA	NA		*	
OTU_143	Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Pseudonocardiaceae	Actinomycetospora			*
OTU_144	Bacteria	Saccharibacteria	NA	NA	NA	NA			*
OTU_145	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Burkholderiaceae	Cupriavidus			*
OTU_146	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Brucellaceae	Pseudochrobactrum	*		
OTU_147	Bacteria	Planctomycetes	Planctomycetia	Planctomycetales	Planctomycetaceae	Blastopirellula		*	
OTU_148	Bacteria	Proteobacteria	Alphaproteobacteria	Caulobacterales	Caulobacteraceae	NA		*	
OTU_149	Bacteria	Proteobacteria	Alphaproteobacteria	Rhodospirillales	Acetobacteraceae	Acidisoma		*	
OTU_150	Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Mycobacteriaceae	Mycobacterium		*	
OTU_151	Bacteria	Proteobacteria	Gammaproteobacteria	Chromatiales	Ectothiorhodospiraceae	NA		*	
OTU_152	Bacteria	Planctomycetes	Planctomycetia	Planctomycetales	Planctomycetaceae	NA		*	
OTU_153	Bacteria	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	NA			*
OTU_154	Bacteria	Proteobacteria	Deltaproteobacteria	Myxococcales	Polyangiaceae	Sorangium	*		
OTU_155	Bacteria	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Chitinophagaceae	NA		*	
OTU_156	Bacteria	Firmicutes	Bacilli	Bacillales	Staphylococcaceae	Staphylococcus		*	
OTU_157	Bacteria	Planctomycetes	Planctomycetia	Planctomycetales	Planctomycetaceae	Planctomyces		*	
OTU_158	Bacteria	Proteobacteria	Gammaproteobacteria	Chromatiales	Ectothiorhodospiraceae	NA		*	

OTU_159	Bacteria	Actinobacteria	Actinobacteria	Solirubrobacterales	Conexibacteraceae	Conexibacter		*	
OTU_160	Bacteria	NA	NA	NA	NA	NA		*	
OTU_161	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Phyllobacteriaceae	Mesorhizobium			*
OTU_162	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Bradyrhizobiaceae	Bosea	*		
OTU_163	Bacteria	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	NA	*		
OTU_164	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Chryseobacterium	*		
OTU_165	Bacteria	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Novosphingobium	*		
OTU_166	Bacteria	Candidatus_Saccharibacteria	NA	NA	NA	NA		*	
OTU_167	Bacteria	Proteobacteria	Alphaproteobacteria	Alphaproteobacteria_incertae_sedis	Rhizomicrobium	NA		*	
OTU_168	Bacteria	Planctomycetes	Planctomycetia	Planctomycetales	Planctomycetaceae	Singulisphaera		*	
OTU_169	Bacteria	division_WPS-1	NA	NA	NA	NA		*	
OTU_170	Bacteria	NA	NA	NA	NA	NA		*	
OTU_171	Bacteria	NA	NA	NA	NA	NA		*	
OTU_172	Bacteria	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Erythrobacteraceae	NA			*
OTU_173	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	Acinetobacter			*
OTU_174	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	Acinetobacter			*
OTU_175	Bacteria	Proteobacteria	Deltaproteobacteria	Myxococcales	Haliangiaceae	Haliangium	*		
OTU_176	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Bradyrhizobiaceae	Afipia		*	
OTU_177	Bacteria	Planctomycetes	Planctomycetia	Planctomycetales	Planctomycetaceae	NA		*	
OTU_178	Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Thermomonosporaceae	NA		*	
OTU_179	Bacteria	Acidobacteria	Acidobacteria_Gp3	Candidatus_Solibacter	NA	NA		*	
OTU_180	Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Geodermatophilaceae	NA		*	
OTU_181	Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Nocardioidaceae	Marmoricola			*
OTU_182	Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Nocardiaceae	Rhodococcus			*
OTU_183	Bacteria	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	Luteibacter	*		
OTU_184	Bacteria	Proteobacteria	Alphaproteobacteria	Rhodospirillales	Acetobacteraceae	Acidisoma	*		
OTU_185	Bacteria	Proteobacteria	Alphaproteobacteria	Rhodospirillales	Acetobacteraceae	NA	*		
OTU_186	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Methylobacteriaceae	Methylobacterium	*		
OTU_187	Bacteria	Bacteroidetes	Bacteroidetes_incertae_sedis	Ohtaekwangia	NA	NA	*		
OTU_188	Bacteria	Proteobacteria	Gammaproteobacteria	NA	NA	NA	*		
OTU_189	Bacteria	Planctomycetes	Planctomycetia	Planctomycetales	Planctomycetaceae	NA		*	

OTU_190	Bacteria	Planctomycetes	Planctomycetia	Planctomycetales	Planctomycetaceae	Aquisphaera		*	
OTU_191	Bacteria	Proteobacteria	NA	NA	NA	NA		*	
OTU_192	Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	NA	NA		*	
OTU_193	Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	NA	NA		*	
OTU_194	Bacteria	Planctomycetes	Planctomycetia	Planctomycetales	Planctomycetaceae	NA		*	
OTU_195	Bacteria	Acidobacteria	Acidobacteria_Gp1	Terriglobus	NA	NA		*	
OTU_196	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Burkholderiaceae	NA		*	
OTU_197	Bacteria	Actinobacteria	Actinobacteria	NA	NA	NA		*	
OTU_198	Bacteria	NA	NA	NA	NA	NA		*	
OTU_199	Bacteria	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Chitinophagaceae	NA			*
OTU_200	Bacteria	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Sinobacteraceae	Alkanibacter			*
OTU_201	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Myroides			*
OTU_202	Bacteria	Proteobacteria	Gammaproteobacteria	NA	NA	NA	*		
OTU_203	Bacteria	Bacteroidetes	Cytophagia	Cytophagales	Cytophagaceae	Spirosoma	*		
OTU_204	Bacteria	Acidobacteria	Acidobacteria_Gp1	Terriglobus	NA	NA	*		
OTU_205	Bacteria	Planctomycetes	Planctomycetia	Planctomycetales	Planctomycetaceae	Blastopirellula	*		
OTU_206	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	*		
OTU_207	Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Thermomonosporaceae	NA	*		
OTU_208	Bacteria	Proteobacteria	Deltaproteobacteria	Bdellovibrionales	Bdellovibrionaceae	Vampirovibrio		*	
OTU_209	Bacteria	Acidobacteria	Acidobacteria_Gp1	Gp1	NA	NA		*	
OTU_210	Bacteria	Verrucomicrobia	Subdivision3	NA	NA	NA		*	
OTU_211	Bacteria	Firmicutes	Clostridia	Clostridiales	NA	NA		*	
OTU_212	Bacteria	Proteobacteria	Gammaproteobacteria	NA	NA	NA		*	
OTU_213	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Cryomorphaceae	NA			*
OTU_214	Bacteria	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	Luteibacter			*
OTU_215	Bacteria	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Sinobacteraceae	Nevskia			*
OTU_216	Bacteria	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Sphingomonas	*		
OTU_217	Bacteria	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Chitinophagaceae	Terrimonas	*		
OTU_218	Bacteria	NA	NA	NA	NA	NA	*		
OTU_219	Bacteria	NA	NA	NA	NA	NA	*		
OTU_220	Bacteria	Verrucomicrobia	Subdivision3	NA	NA	NA		*	
OTU_221	Bacteria	Planctomycetes	Planctomycetia	Planctomycetales	Planctomycetaceae	Singulisphaera		*	
OTU_222	Bacteria	Planctomycetes	Planctomycetia	Planctomycetales	Planctomycetaceae	NA		*	

OTU_223	Bacteria	Verrucomicrobia	Spartobacteria	NA	NA	NA		*	
OTU_224	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Pelomonas		*	
OTU_225	Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	NA	NA		*	
OTU_226	Bacteria	Planctomycetes	Planctomycetia	Planctomycetales	Planctomycetaceae	Aquisphaera		*	
OTU_227	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Beijerinckiaceae	NA		*	
OTU_228	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	Moraxella		*	
OTU_229	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Variovorax		*	
OTU_230	Bacteria	Planctomycetes	Planctomycetia	Planctomycetales	Planctomycetaceae	Aquisphaera		*	
OTU_231	Bacteria	Verrucomicrobia	NA	NA	NA	NA		*	
OTU_232	Archaea	Thaumarchaeota	Nitrososphaerales	Nitrososphaeraceae	Nitrososphaera	NA		*	
OTU_233	Bacteria	Proteobacteria	Deltaproteobacteria	Myxococcales	Polyangiaceae	NA		*	
OTU_234	Bacteria	Planctomycetes	Planctomycetia	Planctomycetales	Planctomycetaceae	Gemmata		*	
OTU_235	Bacteria	Acidobacteria	Acidobacteria_Gp1	Gp1	NA	NA		*	
OTU_236	Bacteria	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Chitinophagaceae	Flavihumibacter			*
OTU_237	Bacteria	NA	NA	NA	NA	NA			*
OTU_238	Bacteria	Saccharibacteria	NA	NA	NA	NA			*
OTU_239	Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	NA	*		
OTU_240	Bacteria	Proteobacteria	Alphaproteobacteria	Alphaproteobacteria_incertae_sedis	Rhizomicrobium	NA	*		
OTU_241	Bacteria	Proteobacteria	Alphaproteobacteria	Caulobacterales	Caulobacteraceae	Caulobacter	*		
OTU_242	Bacteria	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Chitinophagaceae	NA	*		
OTU_243	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	NA	NA	*		
OTU_244	Bacteria	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Chitinophagaceae	Hydrotalea		*	
OTU_245	Bacteria	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Sphingobacteriaceae	Mucilaginibacter		*	
OTU_246	Bacteria	Cyanobacteria	Chloroplast	Chloroplast	Streptophyta	NA		*	
OTU_247	Bacteria	Planctomycetes	Planctomycetia	Planctomycetales	Planctomycetaceae	Aquisphaera		*	
OTU_248	Bacteria	division_WPS-1	NA	NA	NA	NA		*	
OTU_249	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Chryseobacterium		*	
OTU_250	Bacteria	Acidobacteria	Acidobacteria_Gp2	Gp2	NA	NA		*	
OTU_251	Bacteria	Proteobacteria	Alphaproteobacteria	Rhodospirillales	Acetobacteraceae	Acidisoma		*	
OTU_252	Bacteria	NA	NA	NA	NA	NA		*	
OTU_253	Bacteria	Proteobacteria	Deltaproteobacteria	Myxococcales	Cystobacteraceae	NA		*	
OTU_254	Bacteria	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Sphingobacteriaceae	Sphingobacterium		*	*
OTU_255	Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Nocardioidaceae	Nocardioides			*

OTU_256	Bacteria	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Sphingomonas			*
OTU_257	Bacteria	Armatimonadetes	Armatimonadetes_gp5	NA	NA	NA			*
OTU_258	Bacteria	Acidobacteria	Acidobacteria_Gp1	Edaphobacter	NA	NA	*		
OTU_259	Bacteria	Bacteroidetes	NA	NA	NA	NA	*		
OTU_260	Bacteria	Proteobacteria	Deltaproteobacteria	Myxococcales	Haliangiaceae	Haliangium	*		
OTU_261	Bacteria	NA	NA	NA	NA	NA		*	
OTU_262	Bacteria	Proteobacteria	NA	NA	NA	NA		*	
OTU_263	Bacteria	Proteobacteria	Alphaproteobacteria	Caulobacterales	Caulobacteraceae	Phenylobacterium		*	
OTU_264	Bacteria	Proteobacteria	Alphaproteobacteria	Rhodospirillales	Acetobacteraceae	NA		*	
OTU_265	Bacteria	Proteobacteria	Alphaproteobacteria	NA	NA	NA		*	
OTU_266	Bacteria	Verrucomicrobia	Subdivision3	NA	NA	NA		*	
OTU_267	Bacteria	Planctomycetes	Planctomycetia	Planctomycetales	Planctomycetaceae	Gemmata		*	
OTU_268	Bacteria	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Sphingobacteriaceae	NA			*
OTU_269	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Stenothermobacter			*
OTU_270	Bacteria	Proteobacteria	Alphaproteobacteria	Rhodospirillales	NA	NA	*		
OTU_271	Bacteria	Verrucomicrobia	Spartobacteria	NA	NA	NA	*		
OTU_272	Bacteria	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Sphingobacteriaceae	Mucilaginibacter	*		
OTU_273	Bacteria	Proteobacteria	Alphaproteobacteria	Caulobacterales	Caulobacteraceae	Caulobacter	*		
OTU_274	Bacteria	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	NA	*		
OTU_275	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides	*		
OTU_276	Bacteria	NA	NA	NA	NA	NA		*	
OTU_277	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Roseateles		*	
OTU_278	Bacteria	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	Stenotrophomonas		*	
OTU_279	Bacteria	Verrucomicrobia	Subdivision3	NA	NA	NA		*	
OTU_280	Bacteria	Acidobacteria	Acidobacteria_Gp1	Gp1	NA	NA		*	
OTU_281	Bacteria	Proteobacteria	Alphaproteobacteria	NA	NA	NA		*	
OTU_282	Bacteria	NA	NA	NA	NA	NA		*	
OTU_283	Bacteria	Saccharibacteria	NA	NA	NA	NA		*	
OTU_284	Bacteria	Armatimonadetes	Armatimonadia	Armatimonadales	Armatimonadaceae	Armatimonas/Armatimonadetes_gp1		*	
OTU_285	Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Nocardioidaceae	Nocardioides		*	
OTU_286	Bacteria	Bacteroidetes	Cytophagia	Cytophagales	Cytophagaceae	Siphonobacter		*	

OTU_287	Bacteria	Proteobacteria	Alphaproteobacteria	NA	NA	NA		*	
OTU_288	Bacteria	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	Dokdonella			*
OTU_289	Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Corynebacteriaceae	Corynebacterium			*
OTU_290	Bacteria	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Chitinophagaceae	NA	*		
OTU_291	Bacteria	NA	NA	NA	NA	NA	*		
OTU_292	Bacteria	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Novosphingobium	*		
OTU_293	Bacteria	Proteobacteria	Deltaproteobacteria	Myxococcales	Kofleriaceae	Kofleria	*		
OTU_294	Bacteria	Verrucomicrobia	Subdivision3	NA	NA	NA	*		
OTU_295	Bacteria	Armatimonadetes	Chthonomonadetes	Chthonomonadales	Chthonomonadaceae	Chthonomonas/Armatimonadetes_gp3		*	
OTU_296	Bacteria	NA	NA	NA	NA	NA		*	
OTU_297	Bacteria	Planctomycetes	Planctomycetia	Planctomycetales	Planctomycetaceae	Blastopirellula		*	
OTU_298	Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Thermomonosporaceae	Actinoallomurus		*	
OTU_299	Bacteria	Planctomycetes	Planctomycetia	Planctomycetales	Planctomycetaceae	NA		*	
OTU_300	Bacteria	Proteobacteria	Alphaproteobacteria	Caulobacterales	Caulobacteraceae	Brevundimonas		*	
OTU_301	Bacteria	Proteobacteria	Alphaproteobacteria	Alphaproteobacteria_incertae_sedis	Rhizomicrobium	NA		*	
OTU_302	Bacteria	Planctomycetes	Planctomycetia	Planctomycetales	Planctomycetaceae	Blastopirellula			*
OTU_303	Bacteria	Proteobacteria	Alphaproteobacteria	Rhodospirillales	Acetobacteraceae	Acidisoma			*
OTU_304	Bacteria	Proteobacteria	Alphaproteobacteria	Caulobacterales	Caulobacteraceae	Phenylobacterium	*		
OTU_305	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Methylocystaceae	Hansschlegelia	*		
OTU_306	Bacteria	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Chitinophagaceae	NA	*		
OTU_307	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	Rhizobium	*		
OTU_308	Bacteria	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Sphingobacteriaceae	Mucilaginibacter	*		
OTU_309	NA	NA	NA	NA	NA	NA	*		
OTU_310	Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Nocardiaceae	Rhodococcus		*	
OTU_311	Bacteria	Planctomycetes	Planctomycetia	Planctomycetales	Planctomycetaceae	Aquisphaera		*	
OTU_312	Bacteria	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Novosphingobium		*	
OTU_313	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	Acinetobacter		*	
OTU_314	Bacteria	Proteobacteria	Gammaproteobacteria	NA	NA	NA		*	
OTU_315	Bacteria	Planctomycetes	Planctomycetia	Planctomycetales	Planctomycetaceae	Gemmata		*	
OTU_316	Bacteria	Verrucomicrobia	Spartobacteria	NA	NA	NA		*	
OTU_317	Bacteria	Planctomycetes	Planctomycetia	Planctomycetales	Planctomycetaceae	Blastopirellula		*	

OTU_318	Bacteria	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Chitinophagaceae	NA			*
OTU_319	Bacteria	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	NA	NA			*
OTU_320	Bacteria	Saccharibacteria	NA	NA	NA	NA			*
OTU_321	Bacteria	Verrucomicrobia	Verrucomicrobiae	Verrucomicrobiales	Verrucomicrobiaceae	Prosthecobacter	*		
OTU_322	Bacteria	Bacteroidetes	Cytophagia	Cytophagales	Cytophagaceae	Adhaeribacter	*		
OTU_323	Bacteria	Planctomycetes	Planctomycetia	Planctomycetales	Planctomycetaceae	NA	*		
OTU_324	Bacteria	Proteobacteria	Gammaproteobacteria	NA	NA	NA	*		
OTU_325	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Methylobacteriaceae	Methylobacterium	*		
OTU_326	Bacteria	Proteobacteria	Deltaproteobacteria	Myxococcales	NA	NA		*	
OTU_327	Bacteria	Acidobacteria	Acidobacteria_Gp16	Gp16	NA	NA		*	
OTU_328	Bacteria	NA	NA	NA	NA	NA		*	
OTU_329	Bacteria	Proteobacteria	Alphaproteobacteria	Rhodospirillales	NA	NA		*	
OTU_330	Bacteria	_WPS-2	NA	NA	NA	NA		*	
OTU_331	Bacteria	Actinobacteria	Actinobacteria	Acidimicrobiales	Acidimicrobineae_incertae_sedis	Aciditerrimonas		*	
OTU_332	Bacteria	_WPS-2	NA	NA	NA	NA		*	
OTU_333	Bacteria	division_WPS-2	NA	NA	NA	NA		*	
OTU_334	Bacteria	Armatimonadetes	Chthonomonadetes	Chthonomonadales	Chthonomonadaceae	Chthonomonas/Armatimonadetes_gp3		*	
OTU_335	Bacteria	Verrucomicrobia	Spartobacteria	NA	NA	NA		*	
OTU_336	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Bradyrhizobiaceae	Bosea		*	
OTU_337	Bacteria	Saccharibacteria	NA	NA	NA	NA		*	
OTU_338	Bacteria	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	NA	NA			*
OTU_339	Bacteria	Proteobacteria	Gammaproteobacteria	Thiotrichales	Piscirickettsiaceae	Methylophaga			*
OTU_340	Bacteria	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Sinobacteraceae	Alkanibacter			*
OTU_341	Bacteria	Verrucomicrobia	Subdivision3	NA	NA	NA			*
OTU_342	Bacteria	Proteobacteria	Alphaproteobacteria	Caulobacterales	Caulobacteraceae	Asticcacaulis	*		
OTU_343	Bacteria	Acidobacteria	Acidobacteria_Gp4	Gp4	NA	NA	*		
OTU_344	Bacteria	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Novosphingobium	*		
OTU_345	Bacteria	Bacteroidetes	Bacteroidetes_incertae_sedis	Ohtaekwangia	NA	NA	*		
OTU_346	Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Cedecea	*		
OTU_347	Bacteria	Acidobacteria	Acidobacteria_Gp1	Gp1	NA	NA		*	

OTU_348	Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Pseudonocardiaceae	Actinomycetospora		*	
OTU_349	Bacteria	division_WPS-2	NA	NA	NA	NA		*	
OTU_350	Bacteria	Planctomycetes	Planctomycetia	Planctomycetales	Planctomycetaceae	NA		*	
OTU_351	Bacteria	Cyanobacteria	Chloroplast	Chloroplast	Streptophyta	NA		*	
OTU_352	Bacteria	Proteobacteria	Gammaproteobacteria	NA	NA	NA		*	
OTU_353	Bacteria	Actinobacteria	Actinobacteria	Solirubrobacterales	NA	NA		*	
OTU_354	Bacteria	Proteobacteria	Alphaproteobacteria	Rhodospirillales	Acetobacteraceae	NA		*	
OTU_355	Bacteria	Planctomycetes	Planctomycetia	Planctomycetales	Planctomycetaceae	Aquisphaera		*	
OTU_356	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	Acinetobacter		*	
OTU_357	Bacteria	Acidobacteria	Acidobacteria_Gp1	Gp1	NA	NA		*	
OTU_358	Bacteria	Proteobacteria	Alphaproteobacteria	Caulobacterales	Caulobacteraceae	Phenylobacterium		*	
OTU_359	Bacteria	Actinobacteria	Actinobacteria	Solirubrobacterales	Conexibacteraceae	Conexibacter		*	
OTU_360	Bacteria	Bacteroidetes	Cytophagia	Cytophagales	Cytophagaceae	Spirosoma		*	
OTU_361	Bacteria	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus		*	
OTU_362	Bacteria	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Chitinophagaceae	NA			*
OTU_363	Bacteria	Saccharibacteria	NA	NA	NA	NA			*
OTU_364	Bacteria	Proteobacteria	Betaproteobacteria	Nitrosomonadales	Nitrosomonadaceae	Nitrosospira			*
OTU_365	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Myroides			*
OTU_366	Bacteria	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Sphingomonas	*		
OTU_367	Bacteria	Planctomycetes	Planctomycetia	Planctomycetales	Planctomycetaceae	Gemmata	*		
OTU_368	Bacteria	Bacteroidetes	Cytophagia	Cytophagales	Cytophagaceae	Cytophaga		*	
OTU_369	Bacteria	Planctomycetes	Planctomycetia	Planctomycetales	Planctomycetaceae	Gemmata		*	
OTU_370	Bacteria	Planctomycetes	Planctomycetia	Planctomycetales	Planctomycetaceae	Singulisphaera		*	
OTU_371	Bacteria	Armatimonadetes	Armatimonadia	Armatimonadales	Armatimonadaceae	Armatimonas/Armatimonadetes_gp1		*	
OTU_372	Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Sporichthyaceae	Sporichthya		*	
OTU_373	Bacteria	Planctomycetes	Planctomycetia	Planctomycetales	Planctomycetaceae	Aquisphaera		*	
OTU_374	Bacteria	Armatimonadetes	Chthonomonadetes	Chthonomonadales	Chthonomonadaceae	Chthonomonas/Armatimonadetes		*	
OTU_375	Bacteria	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Sinobacteraceae	Alkanibacter		*	
OTU_376	Bacteria	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Chitinophagaceae	Sediminibacterium		*	
OTU_377	Bacteria	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Sphingobacteriaceae	Sphingobacterium		*	

OTU_378	Bacteria	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Sphingomonas		*	
OTU_379	Bacteria	Planctomycetes	Planctomycetia	Planctomycetales	Planctomycetaceae	NA		*	
OTU_380	Bacteria	Acidobacteria	Acidobacteria_Gp3	Gp3	NA	NA		*	
OTU_381	Bacteria	WPS-2	NA	NA	NA	NA		*	
OTU_382	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	Alkanindiges			*
OTU_383	Bacteria	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	Lysobacter			*
OTU_384	Bacteria	Proteobacteria	Gammaproteobacteria	Gammaproteobacteria_incertae_sedis	Solimonas	NA			*
OTU_385	Bacteria	Planctomycetes	Planctomycetia	Planctomycetales	Planctomycetaceae	Aquisphaera			*
OTU_386	Bacteria	Acidobacteria	Acidobacteria_Gp10	Gp10	NA	NA	*		
OTU_387	Bacteria	Acidobacteria	Acidobacteria_Gp4	Gp4	NA	NA	*		
OTU_388	Bacteria	Planctomycetes	Planctomycetia	Planctomycetales	Planctomycetaceae	Pirellula	*		
OTU_389	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Hyphomicrobiaceae	Hyphomicrobium	*		
OTU_390	Bacteria	Proteobacteria	Deltaproteobacteria	Myxococcales	Polyangiaceae	Chondromyces	*		
OTU_391	Bacteria	Actinobacteria	Actinobacteria	Acidimicrobiales	Acidimicrobiaceae	Ilumatobacter	*		
OTU_392	Bacteria	Verrucomicrobia	Spartobacteria	NA	NA	NA	*		
OTU_393	Bacteria	NA	NA	NA	NA	NA		*	
OTU_394	Bacteria	Proteobacteria	Alphaproteobacteria	Rhodospirillales	Rhodospirillaceae	Dongia		*	
OTU_395	Bacteria	Planctomycetes	Planctomycetia	Planctomycetales	Planctomycetaceae	Aquisphaera		*	
OTU_396	Bacteria	Planctomycetes	Planctomycetia	Planctomycetales	Planctomycetaceae	Aquisphaera		*	
OTU_397	Bacteria	Acidobacteria	Acidobacteria_Gp2	Gp2	NA	NA		*	
OTU_398	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	NA	NA		*	
OTU_399	Bacteria	Planctomycetes	Planctomycetia	Planctomycetales	Planctomycetaceae	Aquisphaera		*	
OTU_400	Bacteria	NA	NA	NA	NA	NA		*	
OTU_401	Bacteria	Proteobacteria	Deltaproteobacteria	Bdellovibrionales	Bdellovibrionaceae	Bdellovibrio		*	
OTU_402	Bacteria	Proteobacteria	Alphaproteobacteria	Rhodospirillales	Acetobacteraceae	NA		*	
OTU_403	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Xanthobacteraceae	Pseudolabrys			*
OTU_404	Bacteria	Proteobacteria	Alphaproteobacteria	Rhodospirillales	Acetobacteraceae	NA			*
OTU_405	Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Microbacteriaceae	NA			*
OTU_406	Bacteria	_WPS-2	NA	NA	NA	NA	*		
OTU_407	Bacteria	Acidobacteria	Acidobacteria_Gp3	Candidatus_Solibacter	NA	NA	*		
OTU_408	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Cellvibrio	*		
OTU_409	Bacteria	Gemmatimonadetes	Gemmatimonadetes	Gemmatimonadales	Gemmatimonadaceae	Gemmatimonas	*		

OTU_410	Bacteria	Planctomycetes	Planctomycetia	Planctomycetales	Planctomycetaceae	NA	*		
OTU_411	Bacteria	Acidobacteria	Acidobacteria_Gp1	Gp1	NA	NA	*		
OTU_412	Bacteria	Verrucomicrobia	Subdivision3	NA	NA	NA	*		
OTU_413	Bacteria	Saccharibacteria	NA	NA	NA	NA		*	
OTU_414	Bacteria	Saccharibacteria	NA	NA	NA	NA		*	
OTU_415	Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Thermomonosporaceae	Actinoallomurus		*	
OTU_416	Bacteria	Acidobacteria	Acidobacteria_Gp1	Gp1	NA	NA		*	
OTU_417	Bacteria	Proteobacteria	Alphaproteobacteria	Caulobacterales	Caulobacteraceae	Phenylobacterium		*	
OTU_418	Bacteria	Proteobacteria	Deltaproteobacteria	Myxococcales	Nannocystaceae	Nannocystis		*	
OTU_419	Bacteria	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	NA		*	
OTU_420	Bacteria	Deinococcus-Thermus	Deinococci	Thermales	Thermaceae	Thermus		*	
OTU_421	Bacteria	Armatimonadetes	Armatimonadia	Armatimonadales	Armatimonadaceae	Armatimonas/Armatimonadetes_gp1		*	
OTU_422	Bacteria	Planctomycetes	Planctomycetia	Planctomycetales	Planctomycetaceae	NA		*	
OTU_423	Bacteria	Proteobacteria	Alphaproteobacteria	Rhodospirillales	Rhodospirillaceae	Magnetospirillum		*	
OTU_424	Bacteria	Planctomycetes	Planctomycetia	Planctomycetales	Planctomycetaceae	Aquisphaera			*
OTU_425	Bacteria	Proteobacteria	Deltaproteobacteria	Bdellovibrionales	Bacteriovoracaceae	Peredibacter	*		
OTU_426	Bacteria	Proteobacteria	Gammaproteobacteria	Alteromonadales	Alteromonadaceae	Haliea	*		
OTU_427	Bacteria	Planctomycetes	Planctomycetia	Planctomycetales	Planctomycetaceae	Planctomyces	*		
OTU_428	Bacteria	Proteobacteria	Deltaproteobacteria	Myxococcales	Haliangiaceae	Haliangium	*		
OTU_429	Bacteria	Planctomycetes	Planctomycetia	Planctomycetales	Planctomycetaceae	Aquisphaera		*	
OTU_430	Bacteria	Proteobacteria	Gammaproteobacteria	Pasteurellales	Pasteurellaceae	Haemophilus		*	
OTU_431	Bacteria	Acidobacteria	Acidobacteria_Gp6	Gp6	NA	NA		*	
OTU_432	Bacteria	Proteobacteria	Deltaproteobacteria	Myxococcales	Polyangiaceae	NA		*	
OTU_433	Bacteria	Planctomycetes	Planctomycetia	Planctomycetales	Planctomycetaceae	Gemmata		*	
OTU_434	Bacteria	Firmicutes	Clostridia	Clostridiales	Clostridiales_Incertae_Sedis_XI	Anaerococcus		*	
OTU_435	Bacteria	Acidobacteria	Acidobacteria_Gp4	NA	NA	NA		*	
OTU_436	Bacteria	Proteobacteria	Betaproteobacteria	Neisseriales	Neisseriaceae	Neisseria		*	
OTU_437	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Chryseobacterium		*	
OTU_438	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Cryomorphaceae	Fluviicola			*
OTU_439	Bacteria	NA	NA	NA	NA	NA	*		
OTU_440	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides	*		

OTU_441	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Elizabethkingia	*		
OTU_442	Bacteria	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Sphingomonas	*		
OTU_443	Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Corynebacteriaceae	Corynebacterium	*		
OTU_444	Bacteria	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Sphingobacteriaceae	Sphingobacterium		*	
OTU_445	Bacteria	NA	NA	NA	NA	NA		*	
OTU_446	Bacteria	Saccharibacteria	NA	NA	NA	NA		*	
OTU_447	Bacteria	Proteobacteria	Gammaproteobacteria	NA	NA	NA		*	
OTU_448	Bacteria	Planctomycetes	Planctomycetia	Planctomycetales	Planctomycetaceae	Zavarzinella		*	
OTU_449	Bacteria	Proteobacteria	Deltaproteobacteria	NA	NA	NA		*	
OTU_450	Bacteria	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus		*	
OTU_451	Bacteria	Verrucomicrobia	Opitutae	Opitutales	Opitutaceae	Opitutus		*	
OTU_452	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	NA	NA		*	
OTU_453	Bacteria	Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	Streptococcus		*	
OTU_454	Bacteria	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	Dyella			*
OTU_455	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Hyphomicrobiaceae	Pelagibacterium			*
OTU_456	Bacteria	Planctomycetes	Planctomycetia	Planctomycetales	Planctomycetaceae	NA			*
OTU_457	Bacteria	Firmicutes	Bacilli	Bacillales	Planococcaceae	Lysinibacillus			*
OTU_458	Bacteria	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Erythrobacteraceae	Altererythrobacter	*		
OTU_459	Bacteria	Proteobacteria	Alphaproteobacteria	NA	NA	NA	*		
OTU_460	Bacteria	Proteobacteria	NA	NA	NA	NA	*		
OTU_461	Bacteria	Proteobacteria	Betaproteobacteria	NA	NA	NA	*		
OTU_462	Bacteria	Proteobacteria	NA	NA	NA	NA	*		
OTU_463	NA	NA	NA	NA	NA	NA	*		
OTU_464	Archaea	NA	NA	NA	NA	NA	*		
OTU_465	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides	*		
OTU_466	Bacteria	NA	NA	NA	NA	NA	*	*	
OTU_467	Bacteria	Firmicutes	Clostridia	Clostridiales	Clostridiales_Incertae_Sedis_XI	Peptoniphilus	*	*	
OTU_468	Bacteria	Acidobacteria	Acidobacteria_Gp5	Gp5	NA	NA		*	
OTU_469	Bacteria	Bacteroidetes	Bacteroidetes_incertae_sedis	Ohtaekwangia	NA	NA		*	
OTU_470	NA	NA	NA	NA	NA	NA		*	
OTU_471	Bacteria	Planctomycetes	Planctomycetia	Planctomycetales	Planctomycetaceae	Planctomyces		*	

OTU_472	Bacteria	Proteobacteria	Gammaproteobacteria	NA	NA	NA		*	
OTU_473	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium		*	
OTU_474	Bacteria	Firmicutes	Clostridia	Clostridiales	Clostridiales_Incertae_Sedis_XI	Finegoldia		*	
OTU_475	Bacteria	Planctomycetes	Planctomycetia	Planctomycetales	Planctomycetaceae	Gemmata		*	
OTU_476	Bacteria	Proteobacteria	Deltaproteobacteria	NA	NA	NA			*
OTU_477	Bacteria	Saccharibacteria	NA	NA	NA	NA			*
OTU_478	Bacteria	Cyanobacteria	Chloroplast	Chloroplast	Streptophyta	NA			*
OTU_479	Bacteria	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Chitinophagaceae	NA			*
OTU_480	Bacteria	Planctomycetes	Planctomycetia	Planctomycetales	Planctomycetaceae	NA	*		
OTU_481	Bacteria	Armatimonadetes	Armatimonadetes_gp4	NA	NA	NA	*		
OTU_482	Bacteria	Ignavibacteriae	Ignavibacteria	Ignavibacteriales	Ignavibacteriaceae	Ignavibacterium	*		
OTU_483	Bacteria	Proteobacteria	Deltaproteobacteria	Myxococcales	Polyangiaceae	Jahnella	*		
OTU_484	Bacteria	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Chitinophagaceae	NA	*		
OTU_485	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	*		
OTU_486	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	*		
OTU_487	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Hyphomicrobiaceae	Devosia	*		
OTU_488	Bacteria	Synergistetes	Synergistia	Synergistales	Synergistaceae	Cloacibacillus	*		
OTU_489	Bacteria	Gemmatimonadetes	Gemmatimonadetes	Gemmatimonadales	Gemmatimonadaceae	Gemmatimonas	*		
OTU_490	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	NA	NA	*		
OTU_491	Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Dermabacteraceae	Brachybacterium		*	
OTU_492	Bacteria	Acidobacteria	Acidobacteria_Gp1	Gp1	NA	NA		*	
OTU_493	Bacteria	Actinobacteria	Actinobacteria	Acidimicrobiales	Acidimicrobineae_incertae_sedis	Aciditerrimonas		*	
OTU_494	Bacteria	Firmicutes	Negativicutes	Selenomonadales	NA	NA		*	
OTU_495	Bacteria	Proteobacteria	NA	NA	NA	NA		*	
OTU_496	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	Parabacteroides		*	
OTU_497	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	NA	NA		*	
OTU_498	Bacteria	NA	NA	NA	NA	NA		*	
OTU_499	Bacteria	Verrucomicrobia	Opitutae	Opitutales	Opitutaceae	Opitutus		*	
OTU_500	Bacteria	NA	NA	NA	NA	NA			*
OTU_501	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides			*
OTU_502	Bacteria	Chloroflexi	Thermomicrobia	Sphaerobacterales	Sphaerobacteraceae	Sphaerobacter			*

OTU_503	Bacteria	NA	NA	NA	NA	NA			*
OTU_504	Bacteria	Nitrospirae	Nitrospira	Nitrospirales	Nitrospiraceae	Nitrospira			*
OTU_505	Bacteria	Cyanobacteria	Chloroplast	Chloroplast	Chlorophyta	NA			*
OTU_506	Bacteria	Proteobacteria	Betaproteobacteria	NA	NA	NA			*
OTU_507	Bacteria	Firmicutes	Clostridia	Clostridiales	Clostridiales_Incertae_Sedis_XI	Peptoniphilus	*		
OTU_508	Bacteria	Verrucomicrobia	Verrucomicrobiae	Verrucomicrobiales	Verrucomicrobiaceae	Prosthecobacter	*		
OTU_509	Bacteria	NA	NA	NA	NA	NA	*		
OTU_510	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacterium	*		
OTU_511	Bacteria	Saccharibacteria	NA	NA	NA	NA	*		
OTU_512	Bacteria	Cyanobacteria	NA	NA	NA	NA	*		
OTU_513	Bacteria	Saccharibacteria	NA	NA	NA	NA	*		
OTU_514	Bacteria	division_WPS-1	NA	NA	NA	NA	*		
OTU_515	Bacteria	Verrucomicrobia	Opitutae	Opitutales	Opitutaceae	Opitutus	*		
OTU_516	NA	NA	NA	NA	NA	NA	*		
OTU_517	Bacteria	Bacteroidetes	Cytophagia	Cytophagales	Cytophagaceae	Cytophaga	*		
OTU_518	Bacteria	NA	NA	NA	NA	NA	*		
OTU_519	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	NA		*	
OTU_520	Bacteria	Proteobacteria	NA	NA	NA	NA		*	
OTU_521	Archaea	Thaumarchaeota	Nitrososphaerales	Nitrososphaeraceae	Nitrososphaera	NA		*	
OTU_522	Bacteria	Proteobacteria	Gammaproteobacteria	Legionellales	Coxiellaceae	Aquicella		*	
OTU_523	Bacteria	Verrucomicrobia	Verrucomicrobiae	Verrucomicrobiales	Verrucomicrobiaceae	Roseimicrobium		*	
OTU_524	NA	NA	NA	NA	NA	NA		*	
OTU_525	Bacteria	Acidobacteria	Acidobacteria_Gp1	Gp1	NA	NA		*	
OTU_526	Bacteria	NA	NA	NA	NA	NA			*
OTU_527	Bacteria	Proteobacteria	Deltaproteobacteria	Myxococcales	Polyangiaceae	NA			*
OTU_528	Bacteria	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Sphingobacteriaceae	Pedobacter			*
OTU_529	Bacteria	Deinococcus-Thermus	Deinococci	Deinococcales	Deinococcaceae	Deinococcus			*
OTU_530	Bacteria	Proteobacteria	Betaproteobacteria	NA	NA	NA			*
OTU_531	Bacteria	NA	NA	NA	NA	NA			*
OTU_532	Bacteria	Planctomycetes	Planctomycetia	Planctomycetales	Planctomycetaceae	Schlesneria	*		
OTU_533	Bacteria	NA	NA	NA	NA	NA	*		
OTU_534	Bacteria	Proteobacteria	NA	NA	NA	NA	*		

OTU_535	Bacteria	Proteobacteria	Gammaproteobacteria	Chromatiales	Chromatiaceae	NA	*		
OTU_536	Archaea	Thaumarchaeota	Nitrososphaerales	Nitrososphaeraceae	Nitrososphaera	NA	*		
OTU_537	Archaea	Thaumarchaeota	Nitrososphaerales	Nitrososphaeraceae	Nitrososphaera	NA	*		
OTU_538	Bacteria	Bacteroidetes	Cytophagia	Cytophagales	Cytophagaceae	Fibrella	*		
OTU_539	Bacteria	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Sinobacteraceae	Alkanibacter	*		
OTU_540	Bacteria	NA	NA	NA	NA	NA	*		
OTU_541	Bacteria	NA	NA	NA	NA	NA	*		
OTU_542	Bacteria	Acidobacteria	Acidobacteria_Gp3	Gp3	NA	NA	*		
OTU_543	Bacteria	Gemmatimonadetes	Gemmatimonadetes	Gemmatimonadales	Gemmatimonadaceae	Gemmatimonas	*		
OTU_544	Bacteria	NA	NA	NA	NA	NA	*		
OTU_545	NA	NA	NA	NA	NA	NA	*		
OTU_546	Bacteria	NA	NA	NA	NA	NA	*		
OTU_547	Bacteria	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Novosphingobium	*		
OTU_548	Bacteria	NA	NA	NA	NA	NA	*		
OTU_549	Bacteria	Planctomycetes	Planctomycetia	Planctomycetales	Planctomycetaceae	Aquisphaera	*		
OTU_550	Bacteria	NA	NA	NA	NA	NA	*		
OTU_551	Bacteria	NA	NA	NA	NA	NA	*		
OTU_552	Bacteria	NA	NA	NA	NA	NA	*		
OTU_553	Bacteria	Proteobacteria	Gammaproteobacteria	NA	NA	NA	*		
OTU_554	Bacteria	Acidobacteria	Acidobacteria	Terriglobus	NA	NA	*		
OTU_555	Bacteria	Proteobacteria	Alphaproteobacteria	NA	NA	NA		*	
OTU_556	Bacteria	Planctomycetes	Planctomycetia	Planctomycetales	Planctomycetaceae	NA		*	
OTU_557	Bacteria	Acidobacteria	Acidobacteria	Gp1	NA	NA		*	
OTU_558	Bacteria	Bacteroidetes	Cytophagia	Cytophagales	Flammeovirgaceae	NA		*	
OTU_559	Bacteria	Planctomycetes	Planctomycetia	Planctomycetales	Planctomycetaceae	Pirellula			*
OTU_560	Bacteria	Verrucomicrobia	Verrucomicrobiae	Verrucomicrobiales	Verrucomicrobiaceae	NA			*
OTU_561	Archaea	NA	NA	NA	NA	NA	*		
OTU_562	Bacteria	NA	NA	NA	NA	NA	*		
OTU_563	Bacteria	NA	NA	NA	NA	NA	*		
OTU_564	NA	NA	NA	NA	NA	NA	*		
OTU_565	Bacteria	Proteobacteria	Alphaproteobacteria	Caulobacterales	Caulobacteraceae	Asticcacaulis	*		
OTU_566	Bacteria	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	Stenotrophomonas	*		

OTU_567	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Oxalobacteraceae	Massilia	*		
OTU_568	Bacteria	Proteobacteria	Alphaproteobacteria	Caulobacterales	Caulobacteraceae	Asticcacaulis	*		
OTU_569	Bacteria	Planctomycetes	Planctomycetia	Planctomycetales	Planctomycetaceae	NA	*		
OTU_570	Bacteria	Proteobacteria	Alphaproteobacteria	NA	NA	NA	*		
OTU_571	Bacteria	Actinobacteria	Actinobacteria	Acidimicrobiales	Iamiaceae	Iamia	*		
OTU_572	Bacteria	Proteobacteria	NA	NA	NA	NA	*		
OTU_573	Bacteria	Actinobacteria	Actinobacteria	Bifidobacteriales	Bifidobacteriaceae	Bifidobacterium	*		
OTU_574	Bacteria	NA	NA	NA	NA	NA	*		
OTU_575	Bacteria	NA	NA	NA	NA	NA	*		
OTU_576	Bacteria	NA	NA	NA	NA	NA	*		